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## ROLE OF THE SEXUAL CYCLE IN DEVELOPMENT OF GENOTYPIC AND PHENOTYPIC DIVERSITY IN *Gibberella zeae*

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ROLE OF THE SEXUAL CYCLE IN DEVELOPMENT OF GENOTYPIC AND  
PHENOTYPIC DIVERSITY IN *Gibberella zeae*

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ABSTRACT OF DISSERTATION

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A dissertation submitted in partial fulfillment of the  
requirements for the degree of Doctor of Philosophy  
in the College of Agriculture at the  
University of Kentucky

By

Sladana Bec

Lexington, Kentucky

Director: Dr. Lisa J. Vaillancourt, Professor of Plant Pathology

Lexington, Kentucky

2011

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## ABSTRACT OF THE DISSERTATION

### ROLE OF THE SEXUAL CYCLE IN DEVELOPMENT OF GENOTYPIC AND PHENOTYPIC DIVERSITY IN *Gibberella zeae*

*Gibberella zeae* (anamorph *Fusarium graminearum*) is a homothallic ascomycete pathogen that is responsible for causing Fusarium head blight (FHB) of wheat and small grains. In addition to causing a reduction in yield, harvested grain is frequently contaminated with trichothecene mycotoxins that are harmful for human and animal health. Use of wheat varieties with resistance to FHB is an important strategy to lower its impact. In order to produce varieties with durable resistance, we must understand the origin and degree of genetic diversity present in the pathogen population. In my research, I focused my efforts on an investigation of the role of mating and sexual development in the generation of genotypic and phenotypic variability in *G. zeae*. The goal of one part of my work was to develop new genetic markers that can be used to monitor out-crossing and genetic diversity in the population. I also optimized gene deletion protocols for *G. zeae* so that I could produce mutant and control strains to address my research hypothesis that MAT genes play a direct role in pathogenicity. Application of novel repetitive RFLP probes to a group of *G. zeae* isolates originating from and near Kentucky revealed a surprisingly high degree of diversity in these local populations. Diversity between locations was greater than that within locations, suggesting the relative importance of local inoculum sources. The probes were also useful as genetic markers for segregation analysis. I crossed two genetically closely related, and commonly used, laboratory strains of *G. zeae* and found that this resulted in transgressive segregation for both aggressiveness and toxigenicity. I showed that the very high and very low levels of aggressiveness and toxigenicity in transgressive segregants are heritable. I also showed that selfing produced a higher degree of diversity in these traits among the progeny than was observed among conidial progeny. This suggests the presence of epigenetic factors that impact pathogenicity. Sexual behavior in *G. zeae* is under the control of MATing type genes. I deleted the complete MAT1 locus, and the MAT1-1-1, and MAT1-2-1 genes separately. Deletion of each of the targeted sequences produced the expected shifts in fertility phenotype. The mat1KO strains became asexual, while mat1-1-1KO and mat1-2-1KO strains shifted to obligate heterothallism. Deletion of the MAT1-1-1 and MAT1-2-1 genes had a negative effect on aggressiveness and mycotoxin production *in planta*, but deletion of the complete MAT1 locus had no effect. The set of mutant and ectopic control strains that I generated will be a useful asset that will be made available to the research community.

**KEYWORDS:** *Gibberella zeae*, Fusarium Head Blight, MATing type genes, Ascomycetes, wheat.

Sladana Bec

November 29, 2011

ROLE OF THE SEXUAL CYCLE IN DEVELOPMENT OF GENOTYPIC AND  
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## CHAPTER 1

### Literature Review

#### 1.1. Importance of Fusarium Head Blight disease of wheat and small grains

The fungus *Fusarium graminearum* Schwabe [teleomorph. *Gibberella zeae* (Schwein.) Petch] is the causal agent of Fusarium head blight (FHB) on wheat and small grains, and of Gibberella stalk and ear rot on corn. FHB is one of the most important diseases affecting the production of wheat and barley in the United States and worldwide (Doohan, Brennan, and Cooke, 2003; Miedaner, 1997; Parry, Jenkinson, and McLeod, 1995). Economic losses resulting from the FHB epidemic of 1993 - 2001 devastated farmers and caused socio-economic upheaval in farming communities in the northern plains (Windels, 2000). Producers of spring wheat, soft red winter wheat, barley, and durum wheat lost around 2.5 billion dollars as a result of reductions in grain yield and grain quality during those years (Nganje, Bangsund, and Leistritz, 2002). Losses from FHB have caused a major decline in the number of traditional mid size family farms in Minnesota and North Dakota (Windels, 2000).

FHB results in both quantitative and qualitative losses of harvested grain. Affected grain can become contaminated with trichothecene mycotoxins beyond levels acceptable for human or animal consumption. Deoxynivalenol (DON, aka. vomitoxin) and its acetylated forms (15-ADON and 3-ADON), and nivalenol (NIV), are the *F. graminearum* mycotoxins that pose the greatest threat to human and animal health (Desjardins and Hohn, 1997).

DON affects normal cell function by inhibiting protein synthesis through binding to the ribosomes, and by affecting signal transduction pathways important in cell proliferation, differentiation and apoptosis. Symptoms of DON exposure in animals include nausea, vomiting, fever, weight loss, emesis, reduced fertility, abortion, and stillbirth (Pestka, 2007; Sobrova et al., 2010). Cytotoxic effects of DON have been demonstrated in human primary hematocyte cell cultures (Königs et al., 2008). However, DON has not been shown to be carcinogenic. Among animals, swine are the most sensitive to DON, followed by poultry and ruminants (Rotter, Prelusky, and Pestka, 1996).

The United States Department of Agriculture (USDA), and the Food and Drug Administration (FDA), have set regulatory limits for the concentration of DON present in food and feed. The amount of DON in finished wheat products (germ, bran, and flour) intended for human consumption cannot exceed 1 ppm. DON cannot exceed 10 ppm in grain and grain byproducts, or 30 ppm in distillers and brewer's grains and gluten feeds and meals derived from grains, intended as feed for ruminating beef cattle four months and older. The limit is 5 ppm in grain and grain byproducts intended for swine and dairy cattle feed, with the total amount of contaminated grain not to exceed 20% of the total diet for swine, and 40% of the total diet for other animals (<http://www.fda.gov/Food/GuidanceComplianceRegulatoryInformation/GuidanceDocuments/NaturalToxins/ucm120184.html>, accessed 10-28-2011).

## **1.2 Fusarium Head Blight Disease Cycle**

Onset of a FHB disease epidemic requires the presence of adequate quantities of primary inoculum, a susceptible host, and favorable environmental conditions during the appropriate developmental stage of the host plant. Susceptible cereal genotypes are the most vulnerable to infection at the early stages of anthesis and shortly after (Strange, 1971). The primary inoculum for FHB is produced on plant residues left in the field from the previous season (Sutton, 1982; Goswami and Kistler, 2004). It has been shown that the majority of primary inoculum is comprised of ascospores produced in sexual fruiting bodies (perithecia) on corn, wheat or barley residue (Shaner, 2003). The production of ascospores is dependent on the environment and is optimal under conditions of high humidity and warm temperatures (23° C - 28° C) (Paulitz, 1996). The ascospores are forcibly ejected from the mature perithecia (Trail et al., 2002). Ascospore release occurs as a result of a drop in temperature and increase in humidity. Ascospores are carried by wind currents or splashed by rain droplets onto flowering wheat heads (Sutton, 1982; Reis, 1990; Schmale et al., 2005). It has been proposed that macroconidia are mainly dispersed via water droplets (Parry et al., 1995) while the forcibly ejected ascospores are dispersed more often by wind currents. Thus, ascospores are more important in long distance movement of the pathogen (Doohan et al., 2003; Fernando et al., 1997).

Infection of wheat heads by *F. graminearum* appears to occur via the glumes, following penetration of stomates and colonization of substomatal spaces (Pritsch et al., 2000). Subcuticular growth in inoculated glume epidermal cells is frequently observed at earlier time points. Intracellular growth becomes evident 48-72 hours after inoculation. The intracellular hyphae are very thick and highly branched, in contrast to the extracellular hyphae that are quite thin. These hyphae proceed into the rachis, and from there into the stems via the vascular tissues and pith (Guenther and Trail, 2005).

On a susceptible wheat variety, external symptoms of FHB show up first at the point of infection in the form of water soaking and brown discoloration, and subsequently progress up and down along the rachis. Bleaching and distortion of awns as colonization progresses are often associated with DON production by the fungus and precede senescence of the host tissue (Parry et al., 1995)

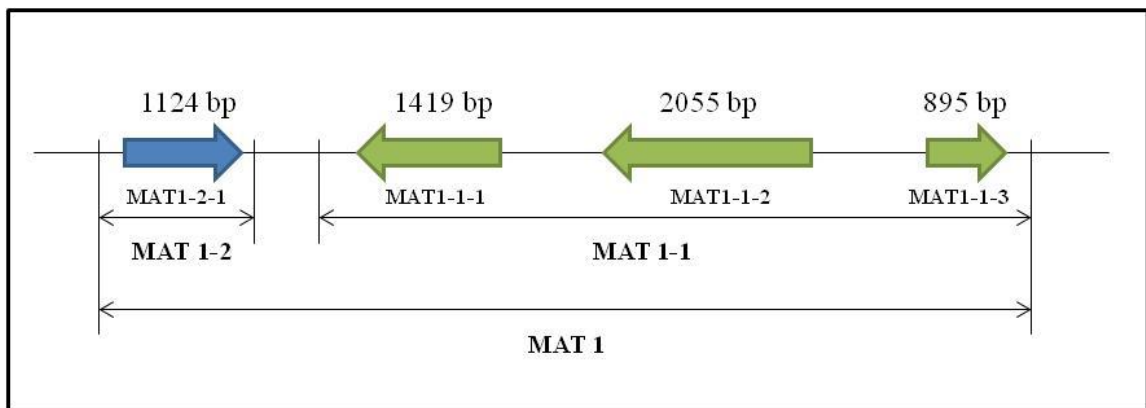
We know relatively little about the specific mechanisms of aggressiveness of *F. graminearum* on wheat. Aggressiveness is quantitative and appears to be conditioned by multiple factors. DON is known to be one aggressiveness factor (Bai, Desjardins, and Plattner, 2002; Proctor, Hohn, and McCormick, 1995). The mycotoxins seem to play a role in helping the fungus to pass from the initially infected floret into the rachis, apparently by preventing the plant from reinforcing its cell walls. Evidence for this comes from studies of a mutant containing a targeted disruption of the *Tri5* gene, which did not produce DON. Despite the fact that DON is generally phytotoxic to plant tissues (Desjardins et al., 2007; Masuda et al., 2007), it does not seem to be important for aggressiveness to barley or corn (Jansen et al., 2005). Recently Boenisch and Schäfer (2011) used a *Tri5* gene promoter fused to a green-fluorescent protein (GFP) reporter gene to investigate the activation of genes responsible for DON production during initial infection events on different floral organs in wheat. They found that trichothecene production is induced in fungal infection structures (foot structures and compound appressoria), however it was not needed for their formation, or for production of normal early symptoms on wheat (Boenisch and Schäfer, 2011). The precise function and role of DON during the process of invasion and colonization of wheat by *F. graminearum* is still unknown.

Perithecial production on intact overwintering plant debris has been directly related to the level of primary inoculum, and thus has a significant impact on the development of FHB epidemics in the following season (Dill-Macky and Jones, 2000). In a cytological study of fungal sexual development during the process of wheat tissue colonization, Guenther and Trail, (2005) observed the presence of two distinct types of hyphae: thinner hyphae with one nucleus per cell; and thicker hyphae that appeared to have two nuclei per cell (dikaryotic). The uninucleate hyphae were found in the pith and along the vascular tissues, while the dikaryotic hyphae were found in the xylem, and also in chlorenchyma tissue associated with perithecial production. Perithecia emerged from the stomata at the wheat internodes and on the upper ridges of the leaf bases in association with silica cells. It was suggested that the dikaryotic hyphae were involved not only in perithecial production, but also in the facilitation of rapid movement of the pathogen through the plant rachis and stem, and additionally that they served as overwintering structures packed with storage lipids to aid in survival between seasons (Guenther et al., 2009). Generally speaking, the production of dikaryotic hyphae in fungi is under the regulation of mating type (MAT) genes. If the MAT genes of *F. graminearum* regulate production of these hyphae, this would give them a direct role in fungal aggressiveness and the development of epidemics.

### **1.3 Sexuality in *Gibberella zeae***

The sexual behavior of *G. zeae* is directly related to its ability to initiate disease epidemics, produce genotypic variability via genetic recombination, and adapt as a population to the environment, including developing resistance to fungicides and overcoming host resistance genes (Bowden and Leslie, 1999; Desjardins et al., 2004; Voss et al., 2010). *G. zeae* is homothallic (self-fertile), with an ability to out-cross when the opportunity arises. Factors that affect out-crossing potential among strains are poorly understood, and the frequency of out-crossing in the field is unknown. However, based on the high variability of neutral markers (Miedaner et al., 2001; Gale et al., 2002; Schmale et al., 2006; Gale et al., 2007) out-crossing is assumed to play an important role in the population structure of *G. zeae* (Leslie and Bowden, 1999).

Proteins encoded by a single MAT locus control sexual reproduction in filamentous ascomycetes. The MAT locus of *G. zeae* is comprised of two mating types, aka. idiomorphs, MAT1-1 and MAT1-2, situated in close proximity within the single MAT1 locus (Yun et al., 2000). The MAT1 locus spans about 7 kb on chromosome 2. The MAT1-1 idiomorph is comprised of three genes, MAT1-1-1, MAT1-1-2, and MAT1-1-3, while the MAT1-2 idiomorph contains only one gene, MAT1-2-1 (Figure 1.1).



**Figure 1.1.** MAT1 locus of *G. zeae* (PH-1). The figure was based on information gathered from <http://www.ncbi.nlm.nih.gov>, accessed 11-08-11.

Each of the idiomorphs encodes a DNA binding protein. In MAT1-1 the DNA binding protein contains an alpha box motif, and in MAT1-2 the DNA binding protein contains a high mobility group (HMG) motif (Coppin et al., 1997, Yun et al., 2000). Both idiomorphs are necessary for homothallism: when either idiomorph was deleted, the resulting mutant became obligately heterothallic (Lee et al., 2003).

Desjardins et al. (2004) deleted the complete MAT1 locus, which rendered the mutant strain sterile. The ability of the mutant strain to induce FHB symptoms on a susceptible wheat variety was unaffected under greenhouse conditions when macroconidia were used as inoculum. However, the mutant strain was unable to initiate a FHB epidemic under field conditions. The explanation given for this was that mutant strain doesn't produce ascospores, and macroconidial dispersal is not efficient enough to allow them to serve as the primary inoculum for an epidemic. It should be noted that there are *Fusarium spp.*

that do not produce ascospores, e.g. *F. culmorum* and *F. poae*, that cause FHB on wheat in Europe (Doohan et al., 2003; Parry et al., 1995). Thus, the necessity for ascospores seems open to question. Another plausible hypothesis to explain the data is that the MAT1 locus itself, as a master regulator of gene expression, is important for pathogenicity.

#### **1.4 Species definitions and phylogenetic diversity in the *F. graminearum* species complex**

*F. graminearum* (*G. zae*) is the primary causal agent of FHB in North America, however a number of other *Fusarium* species cause the same disease in other parts of the world. In Europe, *F. culmorum*, *F. poae*, *F. avenaceum* (teleomorph. *G. avenacea*) and *Microdochium nivale* (teleomorph. *Monographella nivalis*) are all known causal agents of FHB (Sutton, 1982; Parry et al., 1995; Doohan et al., 2003).

*F. graminearum* was previously divided into two groups based on differences in colony morphology and presence or absence of homothallic mating. In 1999, the non-fertile group 2 was renamed *F. pseudograminearum* on the basis of DNA sequence analyses (Aoki and O'Donnell, 1999). Subsequent phylogenetic analysis of a world-wide collection of strains resulted in recognition of *F. graminearum* as a species complex (FSC), consisting of thirteen phylogenetically distinct species, or lineages (O'Donnell et al., 2000; O'Donnell et al., 2004; Starkey et al., 2007).

All of the lineages comprising the FSC produce B-trichothecenes. Based on specific patterns of mycotoxin production, the FSC is divided into three major chemotypes: NIV (producing nivalenol and its acetylated forms); 15-ADON (producing DON and 15-ADON), and 3-ADON (producing a mixture of DON and 3-ADON) (Miller and Greenhalgh, 1991). These chemotypes have been maintained by selection during multiple speciation events. Thus, they may be associated with fitness and provide an adaptive advantage for the pathogen (Ward et al, 2002).

Until recently it was believed that all *F. graminearum* isolates causing FHB in North America were part of a single cosmopolitan population belonging to lineage 7 and the 15-



ADON chemotype (Zeller, Bowden and Leslie, 2003, 2004). However, in 2007 Starkey et al. identified multiple *F. graminearum* isolates belonging to the 3-ADON or NIV chemotypes in Louisiana. In the same year, a new population of 3-ADON strains was identified in the upper Midwest (Gale et al., 2007). Co-existence of 3-ADON and 15-ADON strains was subsequently reported in Manitoba, with 3-ADON strains predominating in the southern part of the province (Guo, Fernando, and Seow-Brock, 2008).

Several lines of evidence suggested that the *F. graminearum* population might be undergoing a shift toward the 3-ADON chemotype. Analysis of two *F. graminearum* strain collections, one isolated between 1980-2000, and the second isolated in 2008, revealed a 15-fold increase in the frequency of the 3-ADON chemotype in the more recent collection. Furthermore, the 3-ADON strains were more aggressive and produced higher levels of mycotoxins than the 15-ADON strains (Puri and Zhong, 2010). Another comparison of the relative aggressiveness and toxin production of 3-ADON and 15-ADON strains showed that 3-ADON strains contaminated susceptible or highly resistant wheat with higher amounts of toxin, however there was no significant difference on moderately resistant wheat varieties (Gilbert et al., 2010). This result led to the rather controversial idea that the current practice of deploying the most resistant wheat varieties to control FHB might be contributing to a shift toward more aggressive strains, and that this shift could be alleviated by using moderately resistant lines instead. These recent findings suggest that the North American population of *F. graminearum* may have more potential for adaptation than we thought. It is important to continue studying the sources and the mechanisms of phenotypic and genotypic diversity relevant to pathogenicity and fitness in this population.

## **1.5 Management of Fusarium Head Blight**

The strategies used to combat FHB include use of fungicides, planting resistant wheat cultivars, crop rotation, agronomic practices (e.g. tillage, crop residue management), and biocontrol (Bai and Shaner, 2004). None of these strategies individually provide sufficient protection (McMullen et al., 2008). Incorporation of multiple strategies in an

integrated crop protection program is the favored approach. For example, crop rotation alone reduced FHB severity by 50%; when combined with the use of a moderately resistant wheat variety, the reduction reached 80%; and when fungicide was also applied at flowering, FHB severity was reduced by over 90% (McMullen et al., 2008).

Fungicides are one of the strategies employed to control FHB and DON accumulation in wheat. The most efficient and consistent results are achieved with triazole fungicides, specifically prothioconazole, metconazole, and tebuconazole+prothioconazole (Paul et al., 2008). However, the level of protection provided by any fungicide is variable, and is directly influenced by the degree of resistance present in the wheat, fungicide efficacy, timing of application, efficiency of coverage, disease pressure, and aggressiveness of the pathogen strain (Mesterházy, Bartók, and Lamper, 2003). Use of fungicide alone often fails to provide sufficient reductions in FHB incidence and DON content during years with high disease pressure (McMullen et al., 2008; Paul et al., 2007). In one study, use of even the most efficacious fungicide (a combination of prothioconazole and tebuconazole) in the absence of other disease protection strategies provided only about a 50% reduction in FHB under high disease pressure (Paul et al., 2008). The effect of fungicide application on DON accumulation varies from reduction, to no significant change, to an actual increase of DON content in the seed (Paul et al. 2007). The reason for this extreme variability is not known.

Use of resistant wheat varieties is a critical component of FHB management. In one study, combining highly resistant wheat varieties and fungicide applications provided a 74% reduction in disease incidence and DON content (Hershman and Bruening, 2011). Sources of resistance to FHB are divided into five types. Type I provides resistance to initial infection; Type II provides resistance to fungal spread through the wheat head; Type III provides resistance to DON accumulation; Type IV provides resistance to kernel infection and damage; and Type V provides resistance to yield loss (tolerance). Type II has been studied most closely, and it has been reported to be more genetically stable than the other types (Bai and Shaner, 2004). In addition to these resistance types, some morphological traits (plant height, absence of awns) are also associated with FHB resistance (Mesterhazy, 1995).

Many wheat-breeding programs use the quantitative trait locus (QTL) associated with Type II resistance that originated from the Chinese variety Sumai 3. Major research effort has been focused on understanding this source of resistance (Liu and Anderson, 2003; Liu et al., 2006; Kianian et al., 2002; Shen, Francki, and Ohm, 2006; Yu et al., 2008). The QTL conferring Type II resistance in Sumai 3 is situated on wheat chromosome arm 3BS, and is considered to be defined by a single gene designated *Fhb1* (Anderson, Chao, and Liu, 2007). It is known that *Fhb1* governs resistance to DON (Lemmens et al., 2005). There are additional QTLs in Sumai 3 on chromosomes 5A, 6B, 3AL, 6AS, and 7D (Anderson et al., 2001; Buerstmayr et al., 2002). The function of these QTLs is unknown.

Relying on single major resistance loci would be expected to increase the rate of adaptation in the pathogen population, hence breeding programs put a lot of effort into combining resistance components from different genetic backgrounds and developing locally adapted varieties with more durable/sustainable resistance (Burlakoti et al., 2009; Kang et al., 2011).

Other sources of resistance used in breeding programs include the moderately resistant varieties Wuhan-1 and Maringa, which carry a QTL for FHB resistance and a QTL that controls DON accumulation (Somers, Fedak, and Savard, 2003). The Brazilian variety Frontana has Type I resistance to initial infection, Type II resistance to fungal spread (Schroeder and Christensen, 1963), and is also thought to have the ability to degrade DON and/or tolerate high levels of DON (Miller, 1986; Wang and Miller, 1988). In my research I used the soft red winter wheat variety 'Truman' developed by the University of Missouri Agricultural Experiment Station and released in 2003. Truman carries quantitative resistance to FHB (McKendry et al. 2005).

Recently there has been increased interest in the possibility of using molecular engineering to create new sources of resistance to FHB. One study found that expression of a fusion protein containing a *Fusarium* - specific antibody and an antifungal peptide in transgenic wheat reduced FHB severity in the greenhouse (Li et al., 2008; Peschen, Li et al., 2004). However, transgenic wheat is still very expensive and difficult to produce and market, so a biotechnological solution to FHB is probably many years in the future.

Significant attention has been given to understanding the role of agronomic crop management techniques (tillage, crop rotation, reduction of overwintering plant debris, planting date) in FHB epidemiology (McMullen et al., 2008; Yuen and Schoneweis, 2007). Continuous wheat and corn-wheat rotations have both been associated with increased FHB disease pressure due to the presence of ample overwintering habitat for the pathogen (Champeil, 2004; Sutton and Vyn, 1990). Dill-Macky and Jones, (2000) reported that yield reduction due to FHB was highest (15%) in a cropping system where wheat followed corn or wheat, and lowest when wheat followed soybeans. The wheat-soybean rotation also had 25% less DON contamination than the wheat following wheat rotation, and 50% less than the wheat-corn rotation.

The same researchers concluded that changes in tillage practices, from conventional (moldboard plowed) to conservation tillage and reduced-tillage systems, contributed to the development of the FHB epidemic in the upper Midwest (Dill-Macky and Jones, 2000).

## **1.6 Questions and Challenges for the Future**

In spite of a great deal of effort expended in combating FHB, it remains as one of the most serious disease problems on wheat and small grains worldwide. The possibility that new, more aggressive, more toxigenic strains of *Fusarium* might emerge that could overcome sources of resistance or fungicides currently used to manage the pathogen is a real threat. We are still in the earliest stages of understanding the biology, and particularly the population biology, of the *Fusarium* species complex that causes FHB. We also don't know enough about the biological mechanisms of fungal pathogenicity. The work described in this dissertation was undertaken to address basic questions about the sources of diversity in this fungus, and factors that affect its pathogenicity. In my Ph.D. research I used a genetic approach, taking advantage of the powerful protocols and resources available for this fungus, to address two aspects of the biology of *F. graminearum*: the role of sexual reproduction and sexual recombination in producing genotypic and phenotypic diversity, specifically focused on pathogenicity and DON production; and the specific role of the MATing type genes in pathogenicity.

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## CHAPTER 2

### **Novel fingerprinting and genetic markers for study of diversity and sexual recombination in *Gibberella zeae* (*Fusarium graminearum*)**

#### **2.1 Introduction**

*Gibberella zeae* (anamorph *Fusarium graminearum*), causal agent of Fusarium head blight (FHB) disease, is one of the most economically important pathogens of wheat and small grains. In the United States (U.S.), losses due to FHB epidemics between 1998 and 2000 totaled two and a half billion dollars (Goswami, and Kistler, 2004; Nganje, Bangsund, and Leistritz, 2002; Nganje, 2003). In addition to reducing total grain yields, the pathogen also produces a variety of trichothecene mycotoxins that are harmful to animal and to human health (Königs, Schwerdt, Gekle, and Humpf, 2008; Sobrova et al., 2010; Wu, Murphy, Cunnick, and Hendrich, 2007).

The group of *Fusarium* genotypes that initiate FHB around the world are classified as members of the *Fusarium graminearum* (*F.g.*) species complex. Due to morphological similarity, the *F.g.* species complex was previously considered to be a single pandemic species. In 2000, O'Donnell et al. applied the concept of genealogical concordance polygenetic species recognition (GCPSR), and recognized seven independent, mostly geographically defined, genetic lineages (phylogenetic species) within the complex (O'Donnell et al., 2000). The latest analysis includes a combination of high-throughput multilocus genotyping assays, GCPSR, and molecular markers and has identified 13 distinct lineages within the *F.g.* species complex, including *F. graminearum* sensu stricto, which comprises lineage 7 (O'Donnell et al., 2004; Ward et al., 2008). A uniting feature among all members of the *F.g.* species complex is the ability to produce type B trichothecene mycotoxins, including deoxynivalenol (DON) and nivalenol (NIV), and their respective acetylated forms 15-ADON and 3-ADON, and 4ANIV and 10ANIV (Brown, Dyer, McCormick, Kendra, and Ronald D Plattner, 2004; Kimura, Tokai, Takahashi-Ando, Ohsato, and Fujimura, 2007; Merhej, Richard-Forget, and Barreau, 2011; Proctor, McCormick, Alexander, and Anne E Desjardins, 2009).

In North America, most isolates causing FHB belong to *F. graminearum* lineage 7, and they produce primarily DON in infected wheat heads in the field, and smaller amounts of 15-ADON as the main acetylated derivative (Cowger et al., 2007). Recently, several genetically distinct subpopulations were characterized by the use of RFLP fingerprinting techniques (Gale et al., 2007). One of these subpopulations produced more 3-ADON versus 15-ADON, and a second produced more NIV versus DON. These 3-ADON and NIV chemotypes could be distinguished from the dominant 15-ADON chemotype by PCR targeting regions of the tricothecene biosynthetic gene cluster. Specific sequence variations within the cluster were absolutely correlated with chemotype (Ward et al., 2002; 2008). The 3-ADON variant was reported to be more aggressive and toxigenic in greenhouse tests, and it had a higher growth rate and fecundity *in vitro*, although it is not known if these traits are a direct result of its chemotype (Gale et al., 2007; Ward et al., 2008).

The degree of pathogenically-significant phenotypic and genotypic variation that exists in the North American population of *F. graminearum* has important implications for the durability of FHB resistance breeding efforts. Interregional movement of people, contaminated wheat seeds, and other plant materials will inevitably bring members of previously isolated pathogen subpopulations together. We don't know whether members of other regional 15-ADON subpopulations may be more fit, more toxigenic, or more aggressive in the field on wheat bred for resistance against indigenous pathogen subpopulations. We do know that there is significant quantitative variation among 15-ADON isolates in many pathogenically significant traits (Gilbert et al., 2001; Walker et al., 2001; Goswami and Kistler, 2005; Akinsanmi et al., 2007). It has been suggested that the North American population consists of a single interbreeding group of genetically and phenotypically diverse isolates, and that there are essentially no regional differences in allelic frequencies (Bowden and Leslie, 1992; Dusabenyagasani et al., 1999; Walker et al., 2001; Zeller et al., 2003, 2004). The fungus is homothallic, and a high degree of genetic polymorphism has been reported in the population (Bowden and Leslie, 1992; Dusabenyagasani et al., 1999; Walker et al., 2001; Zeller et al., 2003, 2004). However, there appears to be only limited variation among regional subpopulations in relative allelic frequencies (Zeller et al., 2003; 2004). Thus, it is generally believed that resistant

wheat varieties bred using local isolates for screening will remain effective even if strains from other regions of North America are introduced, or if novel strains are produced by sexual recombination (Zeller et al., 2003; 2004; Cowger et al., 2007). This belief has not yet been rigorously tested, although data have recently been presented in support of a hypothesis that an adaptive shift to the more aggressive 3-ADON chemotype may be occurring in the pathogen population in North America (Ward et al., 2008).

The success of plant breeding strategies to combat FHB epidemics depends on our ability to monitor and measure the degree of genetic diversity in pathogen populations over time. Diversity in *F. graminearum* has been measured using a variety of molecular markers including sequence characterized amplified regions (SCAR) (Carter et al., 2002); randomly amplified polymorphic DNA (RAPD) (Jana et al., 2003); amplified fragment length polymorphisms (AFLP) (Alvarez et al., 2010; Leslie and Bowden, 2008); restriction fragment length polymorphisms (RFLP) (Gale et al., 2002); simple sequence repeats (SSR) (Singh et al., 2011); single nucleotide polymorphisms (SNP) (Lysøe, Seong, and Kistler, 2011; Yang et al., 2008); and variable number of tandem repeats (VNTR) (Suga, Gale, and Kistler, 2004). Each of these marker types has advantages and disadvantages, but all of them are relatively time-consuming to develop and characterize. With the public availability of a whole genome sequence for *F. graminearum* (Cuomo, 2007), the process of identification and design of novel molecular markers can be accelerated. In my research I used CAPs and repetitive RFLP markers.

Kentucky represents the transition between the Northern and Southern corn belts, and between the Eastern and Western corn belts, and also between the Mid-Atlantic and Midwestern wheat production areas. It is potentially a hot spot for mixing among genotypes of *F. graminearum* that are adapted to the surrounding wheat and corn producing regions. The half million acres of soft red winter wheat (SRWW) produced annually in Kentucky represents a critical source of capital for grain producers in the state. Nearly entire Kentucky winter wheat crop is planted in a rotation following corn, an agronomically efficient practice that allows growers to take advantage of the entire growing season. Gibberella stalk and ear rot, also caused by *F. graminearum*, are common in the corn crop, and the resulting pervasiveness of *F. graminearum*, combined with the prevalence of no-till or minimal tillage cropping systems, puts Kentucky wheat

at high risk for FHB. Resistance-breeding programs for varieties adapted to Kentucky and the southeastern U.S. are currently underway at the University of Kentucky (U.K.). The screening program at U.K. relies on field inoculation with a combination of several indigenous isolates of *F. graminearum*, all of which belong to the lineage 7, 15-ADON type.

The diversity of *F. graminearum* has not been much explored in Kentucky. In the current study I characterized the genotypic and chemotypic diversity among a group of *Fusarium* strains isolated from symptomatic wheat heads collected from across the state, as well as among a group of strains used in the U.K. breeding program over several seasons. I identified repetitive elements in the sequenced genome of strain PH-1 and used them to design novel RFLP fingerprinting markers that were used to evaluate genotypic diversity. I tested the applicability of the same markers for analysis of genetic crosses. For this purpose, I employed molecular tools to mutate the mating type genes and shift the reproductive system of the fungus from homothallic to obligate heterothallic, as previously described by Lee et al. (2003). Progeny from a cross between  $\Delta\text{mat1-1-1}$  (Gz3639) (Lee, Leslie, and Bowden, 2008) and  $\Delta\text{mat 1-2-1}$  (PH-1) provided a sample for testing the use of the novel RFLP probes as genetic markers.



## 2.2 Materials and Methods

**Isolation and culture of fungal strains:** The fungal isolates used in this study were recovered from wheat heads displaying symptoms of FHB. The symptomatic wheat samples were collected from four locations in Kentucky: Lexington, in Fayette Co. (2 fields); Princeton, in Caldwell Co. (2 fields); Owensboro, in Daviess Co. (1 field); and one field in Logan Co. Samples were also collected from one location in Indiana (Hatfield, in Spencer Co.) across the Ohio River from Owensboro (Figure 2.1). Between four and six wheat heads were collected from each location. Samples were disinfested in 10% bleach with agitation for 10 minutes, followed by three washes in sterile water. Disinfested plant tissues were dried by blotting on sterile paper towels. Small sections were taken from the edges of symptomatic lesions and plated on acidified potato dextrose agar (PDA) (1 mL of 85% lactic acid per 1 L of PDA). All isolates were single-spored and stored on silica at -80°C (Tuite, 1969, after Perkins, 1963). All strains were routinely cultured on PDA at 23° C under constant light. Isolates were grown on acidified PDA to check for the production of auro- and rubrofusarin pigments characteristic of *Fusarium graminearum* strains. Asexual spores for wheat inoculations were produced by growing the isolates for 7 days at 23° C and continuous light on mungbean agar media (Bai and Shaner, 1996).

**DNA extraction:** For DNA extraction, 5 ml of YEPD medium (20 g dextrose, 20 g bacto-peptone, 10 g yeast extract) was inoculated with an 8-mm agar plug taken from the edge of an actively growing colony. Cultures were incubated at 25° C for 5-7 days at 250 rpm. Mycelia were harvested by decanting cultures onto sterile paper and blotting the excess liquid. Mycelia were flash-frozen in liquid nitrogen and stored at -80° C until extraction. Frozen mycelia were lyophilized and then pulverized in individual 2 ml Eppendorf tubes by using a mini-pestle, or in 96-deep well plates by using a 2000 GENO Grinder (Spex Cretiprep) (500 strokes/sec for 30 sec). One ml of warm lysis buffer (0.5 M NaCl, 1% SDS, 10 mM Tris HCl, Ph7.5, 10 mM EDTA) was added per 100-200 mg of pulverized fungal tissue, and the samples were incubated at 65° C for 30 min, vortexing once during the incubation. After incubation the samples were transferred into individual

tubes containing 660 µl PCI (25 parts phenol, 25 parts chloroform, 1 part iso-amyl-alcohol), mixed by inverting 4-6 times, then incubated at 65° C for an additional 30 min. The contents were mixed once again during incubation. The samples were centrifuged in a tabletop centrifuge for 20 min at maximum speed to separate the phases. DNA was precipitated from the aqueous phase by using 1 volume of isopropanol, and the pellet was washed twice with 70% ethanol. The pellet was resuspended in 100 µl of TE, pH 7.9, and 2 µl of a 5-mg/ml concentration of RNase A, at 65° C for 1 h. Between 1 and 5 µg of each DNA sample was digested with appropriate restriction enzymes (RE) and used in Southern hybridizations (Southern, 1975) for evaluation of transformant and wild-type strains.

**Chemotyping PCR:** The chemotype determination was done using the multiplex PCR screen published by Ward et al, 2002 that differentiates between the 15-ADON and 3-ADON chemotypes by targeting portions of the Tri3 and Tri12 genes. A combination of the 3CON/3D15A/3D3A oligomer primers (Table 2.1) was used to amplify diagnostic bands, 610 bp for the 15-ADON chemotype and 243 bp for the 3-ADON chemotype. About 50ng of genomic DNA was used as a template. PCR amplification reactions contained Phusion polymerase (Finnzymes F-530S) and other components according to the manufacturer's instructions. The thermocycling protocol consisted of initial denaturation for 3 min. at 94° C; followed by 35 cycles of 30 sec denaturation at 94° C, 20 sec annealing at 62° C, and 45 sec extension at 72° C; and one extension cycle for 7 min. at 72° C. A sample of PCR reaction was electrophoresed on 0.8% agarose gel and visualized under UV light.

### **Generation of molecular probes**

**Species-specific PCR marker:** The Fg16 primer pair that amplifies a *F. graminearum* complex-specific sequence (Demeke et al, 2005) was used to confirm species identity for each isolate. About 50 ng of genomic DNA was used as a template in a PCR reaction. PCR amplification reactions contained Phusion polymerase (Finnzymes F-530S) and other components according to the manufacturer's instructions. The thermocycling protocol consisted of initial denaturation for 3 min. at 94° C; followed by 35 cycles of 30

sec denaturation at 94° C, 20 sec annealing at 62° C, and 30 sec extension at 72° C; and one extension cycle for 7 min. at 72° C. A sample of PCR reaction was electrophoresed on 0.8% agarose gel and visualized under UV light.

**CAPS markers:** Cleaved Amplified Polymorphic Sequences (CAPS) markers were generated by using a list of single-nucleotide polymorphisms (SNPs) differentiating PH-1 and Gz3639 (Cuomo et al., 2007). The list of SNPs was downloaded from ([http://www.broadinstitute.org/annotation/genome/fusarium\\_graminearum/MultiDownloads.html](http://www.broadinstitute.org/annotation/genome/fusarium_graminearum/MultiDownloads.html)). Four unlinked SNPs that spanned *EcoRI* and *PstI* restriction sites were identified, and a ~500 bp segment spanning each selected SNP site was amplified by using primer sets that were designed manually for each region (Table 2.1). PCR amplification reactions contained Phusion polymerase (Finnzymes F-530S) and other components according to the manufacturer's instructions. The thermocycling protocol consisted of initial denaturation for 1 min at 94° C; followed by 35 cycles of 30 sec denaturation at 94° C, 20 sec annealing at 68° C, and 45 sec extension at 72° C; and one extension cycle for 7 min. at 72° C. Three µL of each PCR amplicon was used for each restriction reaction. Restriction reactions used Invitrogen restriction enzymes *EcoRI* (Invitrogen Cat #15202-015) and *PstI* (Invitrogen Cat. # 15215-015) according to the manufacturer's instructions. Restriction reactions were separated on a 1% agarose gel for analysis.

**Telomere marker:** A telomere probe was generated by using two oligomer primers (Table 2.1). The probe was synthesized by performing PCR without a template, resulting in generation and amplification of primer dimers and very large increases in product size in the later cycles (Schechtman, 1990). Phusion polymerase, which has proofreading ability, was used for the PCR. Twenty pmol of each primer was added to each PCR reaction mix with the manufacturer's recommended buffer. The thermocycling protocol consisted of 35 cycles of denaturation for 30 sec. at 94° C, annealing for 30 sec. at 50° C and extension for 60 sec at 72° C; followed by one 2-min. extension at 72° C. PCR products were separated on 1% agarose gel. DNA with a size range of between 1.5 and 2 Kb was excised and purified with a gel extraction kit (QIAquick®, Quiagen cat.#28704).

The purified product (50-80 ng) was labeled using the Prime-a-gene labeling system (Promega, cat.#U1100) and used for Southern hybridization analysis (Southern, 1975).

**Novel fingerprinting probes:** Repetitive motifs present in the genomic DNA sequence of *Gibberella zeae* strain PH-1 were identified by blasting the whole genome sequence of PH-1 against itself. Two repetitive motifs were selected and polymerase chain reaction (PCR) primers were designed to amplify ~ 500 bp regions surrounding the sequences. Resulting PCR amplicons were gel-purified and labeled with radioactive isotope <sup>32</sup> P for use in Southern hybridization analysis. Five hundred ng of the genomic DNA isolated from the fungal strains was digested with the restriction enzyme *BsoBI*. The DNA fragments were separated on 0.8% agarose gel, transferred to a charged nylon membrane, and probed with the radioactively labeled probes. The PCR primers used to amplify the probes are listed in Table 2. The PCR conditions used to create the probes consisted of 3 minutes of denaturation at 94° C; followed by 35 cycles of denaturation for 30 sec at 94° C, 15 sec of annealing at 55° C, and 40 seconds of extension at 72° C; and a final extension of 7 min at 72° C.

**Analysis of RFLP data:** The RFLP data was coded as presence ("1") or absence ("0") of the signal and recorded in a matrix set. Each RFLP locus is defined as a combination of a probe and a restriction enzyme. The total sample set was clone-adjusted by including only a single clone for each unique fingerprint in the analysis. The data matrix was analyzed by using the Statistical Analysis Software (SAS) package to generate Jaccard's similarity coefficients for the construction of dendograms using the unweighted pair group method with arithmetic average (UPGMA) procedure.

**Sexual crosses:** The parental strain mat1-1-1KO(Gz3639) is a MAT1-1-1 deletion strain created in Dr. John Leslie's lab in KSU and it was provided to us by Dr. Frances Trail (Appendix 1 of this Dissertation). The other parental strain, mat1-2-1KO(PH-1), is a MAT1-2-1 deletion mutant produced by split-marker targeted gene deletion in the PH-1 background (Chapter 4 of this Dissertation). The parental strains were crossed by using

the mycelial plug method (Bowden and Leslie, 1999). Five-mm plugs of mycelia of each parental strain, taken from the edges of actively growing cultures on potato dextrose agar (PDA), were placed on opposite halves of a 60 mm Petri plate. After four days of incubation at 23° C, perithecial production was induced by applying 500-1000 µl of 2.5% Tween 60 to the surface of each plate, and gently rubbing the aerial mycelium with a sterile glass rod to flatten it. Following induction, the plates were incubated at 23° C with constant fluorescent light until perithecial maturation. The mature perithecia extruded cirrhi that contained hundreds of ascospores 2-3 weeks after induction. A sterile glass needle was used to pick up individual cirrhi, which were then dispersed in water and plated on 2% water agar. After 10-14 hours of growth on water agar, isolates arising from single ascospores were transferred to individual 6 mm Petri plates.

**FHB pathogenicity assay:** Pioneer 2555 (susceptible), Pioneer 25R18 (type II resistance), and "Truman" (native quantitative resistance, moderately resistant), SRWW varieties were used for this study. Wheat seeds were planted in a mixture of topsoil (Maury silt loam) and PromixBX growth substrate (3:2) in a 72-cell format at the rate of three seeds per cell. The seeds were lightly covered with a moist layer of soil mixture. Seeds were germinated in the greenhouse for 3-5 days at ambient temperature of 25 °C, and 12 h photoperiod. Shortly after germination and seedling emergence, seedlings were treated with the systemic fungicide NOVA<sup>TM</sup> 40W (Dow AgroSciences Canada Inc.) at the recommended rate for powdery mildew, and transferred to a cold room (~4 °C) with constant fluorescent light for 8 weeks. At the end of the vernalization period, the seedlings were transplanted in cone containers filled with a mixture of topsoil (Maury silt loam) and PromixBX (3:2). Transplanted wheat plants were grown in greenhouse with a 14/10 photoperiod, provided by "Hortilux" LU430S/HTL/EN high pressure sodium lights, and ambient temperatures between 25-28°C. Transplants were fertilized with 150 ppm of N:P:K (20:10:20) fertilizer formulation every 14 days starting two weeks after transplanting, with the last fertilization at heading. Flowering typically occurred after 3-4 weeks. At early- to mid- anthesis, a single centrally positioned floret on the primary flowering stem of each plant was inoculated with 10 µl of a spore suspension as

described by (T Miedaner, Moldovan, & Ittu, 2003). Symptom severity was recorded at seven and ten days post-inoculation, as the number of FHB symptomatic spikelets per inoculated spike.

The strains used for inoculation were re-isolated from the symptomatic florets and used to repeat infection on the same variety to complete Koch's postulates.

## 2.3 Results

**Characterization of fungal isolates:** A total of 82 *Fusarium* isolates were recovered from mature heads of soft red winter wheat with symptoms of FHB collected from six production fields across the region, and from the U.K. experimental farm in Lexington (LEX2) (Figure 2.1, Table 2.2). All of the strains were collected in the spring of 2007. The strains were tentatively identified as *F. graminearum* on the basis of their macroconidial and colony morphology.

All of these isolates, together with a set of 18 isolates obtained from the U.K. wheat breeding program, were evaluated by using species-specific and chemotype PCR primer sets (Demeke et al, 2005; Ward et al, 2002). The identities of all amplicons were confirmed by sequencing. All but five of the isolates produced the expected amplification product with the *F. graminearum* species-specific primers, confirming their identity as *F. graminearum* (Figure 2.2). The five exceptional isolates were all recovered from symptomatic wheat heads collected in Owensboro. Although products of the expected size were amplified from four of these strains, the sequences of the amplification products did not match *F. graminearum* (Appendix 2 of this dissertation). All except the same five isolates produced an amplicon specific to the 15-ADON chemotype with the chemotype primers (Figure 2.3). Three of the five exceptional strains did produce an amplicon with the chemotype primers that was similar in size to that expected for 3-ADON isolates, but its sequence was unrelated to the *Tri12* gene that is the target of these primers (Ward et al, 2002) (Appendix 2 of this dissertation).

Genomic DNA from each of the strains was restricted and probed with a telomere-specific probe (TTAGGG)<sub>n</sub> (Figure 2.4). *F. graminearum* has four chromosomes, and

thus we would expect to see no more than eight bands hybridizing to the telomere probe. The five exceptional isolates from Owensboro could be separated into three groups based on the patterns of hybridization, one group with 8 hybridizing bands, one with 14 bands, and another very similar one with 15 bands. These patterns were distinct from the other strains, among which 32 different hybridization patterns could be discerned, indicating a surprisingly large amount of diversity. None of the other strains produced more than 8 bands, and most produced fewer, probably due to the presence of doublets. Patterns were shared more frequently among strains from the same locations than among strains from different locations. The most diversity, consisting of seven different hybridization patterns, was found among 12 isolates collected in Hartfield, IN (Table 2.2). The least diversity was found among the two confirmed *F. graminearum* strains from Owensboro, although this could be a function of the very small sample size. Isolates from Logan County also had relatively low levels of diversity, with only two patterns found among the eight isolates (Table 2.2).

#### **RFLP fingerprint diversity of *Fusarium* isolates within and between populations:**

The Rep1.92 RFLP multilocus probe hybridized to 24 polymorphic bands, while the Rep1.52 probe hybridized to 2 polymorphic bands (Figures 2.5, 2.6). Neither probe hybridized to the five exceptional strains from Owensboro.

The RFLP probes revealed a strikingly large amount of genotypic diversity among the isolates. The percentage of isolates with unique RFLP fingerprints in each location ranged from 37.5% of the isolates from Logan County, to 100% of the isolates from Owensboro (Table 2.2). Among the isolates used as an inoculum mixture by the U.K. wheat-breeding program, 94% had unique RFLP fingerprints.

Multiple genotypes were often isolated from the same wheat head. The number of heads harboring multiple genotypes ranged from zero in the collections from Owensboro and Logan County, to six (100% of the heads) in the LEX4 location (Table 2.2). Three of the six heads from the LEX4 collection contained three different genotypes, while the other three harbored two genotypes each.

There were no isolates with identical RFLP fingerprints from different locations. Thus, every location appeared to have a unique population.

Each unique genotype was scored for presence or absence of the polymorphic bands, and the output was used to construct Unweighted Pair Group Method with Arithmetic Mean (UPGMA) distance dendrograms (Figures 2.7-2.9). The UPGMA analysis presented in Figure 2.7 was generated from RFLP data of isolates collected in 2007. The isolates from the U.K. wheat-breeding group were excluded from this analysis, although the isolates collected from the border surrounding the artificially inoculated FHB disease nursery (LEX2) are included. Clustering appears to be generally related to the origin of the genotypes, with those from the same location grouping most closely together in most cases. There are two major groups, separated by an average distance of 0.98. The first group is comprised of the genotypes from Princeton and the LEX2 isolates. The second group contains the genotypes from Owensboro; Hartfield, IN; Logan County; and the LEX4 collection. The OKY2 genotypes are located within a cluster that also contains the HIN1 genotypes from just across the Ohio River. The genotypes from Logan County seem to be somewhat diverse, with one clustered with the OKY2 and HIN1 genotypes, and the other grouping with the LEX4 genotypes. LEX2 genotypes also seem to be somewhat variable, with one clustering with the PKY5 group, and another clustering with the PKY6 group.

Figure 2.8 shows the relationships among *F. graminearum* genotypes comprising the inoculum mixture used by U.K. wheat-breeding group over the past several years. The group of isolates used as inoculum is diverse, with 94% of them having unique fingerprints. Unfortunately, we do not have information on the precise origin of the isolates, although all were isolated in Kentucky. There appears to be no relationship between fingerprints and the years that the isolates were collected.

A combined analysis of all of the genotypes included in this study resulted in the same two major clusters, with LEX2 genotypes grouping with PKY5 and PKY6 isolates in one cluster, and LEX4, OKY2, HIN1, and LOKY3 in the second cluster (Figure 2.9). The DVS genotypes were distributed through both of these major groups, not an unexpected result given that these isolates are collected from across the state. Isolates from the DVS



collection are used to inoculate the FHB nursery at the Spindletop experimental farm in Lexington. The LEX2 group of isolates was collected from symptomatic wheat heads in the border planted around this nursery in 2007. However, the dendrogram presented in Figure 2.9 does not show an obvious relationship between the fingerprints of isolates from the DVS and LEX2 collections. The LEX2 isolates are similar to some isolates from the DVS collection, but not to others.

### **Characterization of the Owensboro isolates**

Results of the PCR, telomere probe, and RFLP fingerprinting tests all suggested that five *Fusarium* isolates from Owensboro were not *F. graminearum*. On closer examination, there appeared to be a slight difference in macroconidial shape and the morphology of the tip cell, which lacks the *F. graminearum*-specific tapering. The foot cell also was not very distinct (Figure 2.10a,b). However, these differences were very subtle, and there was no difference in macroconidial size.

The isolates were sent to Dr. Todd Ward at the USDA Mycotoxin Laboratory in Peoria for further tests. Dr. Ward and his team ran the isolates through their MLGT assay, which identifies species and trichothecene chemotype based on SNPs (Ward et al. 2008). This analysis confirmed that these strains were not members of the B-trichothecene group of *Fusarium* spp., to which *F. graminearum* belongs. They sequenced a portion of the elongation factor 1 (EF1) gene for each isolate and compared those sequences via BLAST on the Fusarium ID site (Appendix 2 of this Dissertation). The closest match for two of the strains (OKY2-2.1B3 and OKY2-2.1C1) was a *F. tricinctum* species complex (FTSC) strain that has recently been defined as *F. acuminatum*. Two others (OKY2-2.2B1 and OKY2-2.2B2) also appeared to be members of the FTSC, but did not match any known species, and thus may be undescribed.

One of the novel strains, OKY2-2.2B1, was used to inoculate a line of SRWW that is highly susceptible to FHB (Pioneer 2555) in the greenhouse. This strain did not produce FHB symptoms. The initially infected floret became brown and discolored, resembling a Type II resistance response (Figure 2.10c, d).

**Segregation of RFLP Markers:** To test whether hybridizing bands identified by the RFLP fingerprinting probes are linked or allelic, a cross was made between a  $\Delta$ mat1-1-1 strain (generated in the Gz3639 background) and a  $\Delta$ mat1-2-1 strain (generated in the PH-1 background).

Figure 2.11 and Table 2.3 present the results of segregation analysis of the four polymorphic bands that hybridize to the RFLP probe Rep 1.92 in the two parental strains. The bands were scored for presence or absence among 20 progeny of the cross between the PH-1 and Gz3639 MAT mutants. All four markers segregated in the expected 1:1 ratio, with  $\chi^2 = 0.4, 1.6, 0.4, \text{ and } 1$ , respectively and with an associated P-value = 0.99 for all of the markers. Also, all of the markers segregated independently in all possible combinations in a 1:1:1:1 ratio. The  $\chi^2$  and associated P-values are reported in Table 2.3.

## 2.4. Discussion

Genetic and phenotypic diversity in pathogen populations results from migration and gene flow, sexual recombination, and mutation. Selective forces, including host factors such as resistance, act on this diversity to drive shifts in the pathogen population (Burdon and Silk, 1997). The ability to track genetic diversity in a pathogen population is an important aid to understanding the role of selective forces in the development of disease epidemics. For example, shifts in the population may indicate selection by sources of resistance or fungicide regimes, thus providing data that could help to predict and possibly prevent future epidemics. The frequency of out-crossing in the population will affect the speed with which novel hybrid genotypes may arise in the populations. A better understanding of the factors that affect the frequency of out-crossing is needed. Availability of polymorphic, neutral genetic markers is essential in order to monitor genetic diversity and recombination.

In this chapter I have taken advantage of the recently published sequences of two strains of *G. zeae* (Cuomo et al., 2007) to develop some useful classes of markers for genetic studies. Cleaved amplified polymorphism (CAP) markers have been used previously for genetic analysis of *G. zeae* (Gale et al., 2005). CAPs are generated by PCR amplification of a region surrounding a SNP. The resulting amplicon is restricted using a suitable restriction enzyme that discriminates between the two samples based on the SNP. I utilized a published list of SNPs between strains PH-1 and Gz3739 to develop some CAP markers (Cuomo et al., 2007). CAPs were very useful as segregating genetic markers to detect recombination among progeny derived from a cross of PH-1 and Gz3639. Analysis of CAP markers is fast and technically simple, but they do have some limitations. Since they are generated by the use of PCR, they are inherently prone to PCR artifacts, even with polymerases with proofreading ability. Optimization, validation (e.g. sequencing PCR products), and replication are needed to reduce the possibility of artifacts. Several other types of PCR-based markers have been developed for analysis of *G. zeae*, among them the species-specific PCR primers that are reported to amplify only isolates of *G. zeae* (Demeke et al, 2005) and PCR primers that are reported to produce chemotype-specific products (Ward et al., 2002). When I used these published primers to analyze a group of isolates from Kentucky, I found that they were also prone to artifacts: amplified

products of the correct size did not necessarily have the correct sequence, when they were checked.

A CAP marker detects polymorphism at only one locus, hence multiple CAP markers have to be evaluated to generate enough data to address a hypothesis. The time and the price per single locus scored make them an expensive option. Repetitive “fingerprinting” probes for RFLP are more useful because they can identify more than one polymorphic band in a single experiment, and they are generally less prone to artifacts than PCR-based methods. I utilized a published telomere probe that detects polymorphic chromosome ends, and found that it did reveal diversity among strains, but because *G. zeae* has only four chromosomes, the number of hybridizing fragments produced was small, and thus resolving power was limited. The PH-1 genome sequence once again proved to be a useful resource: by comparing the genome sequence against itself, Dr. Mark Farman was able to generate a list of repetitive sequences that had potential as fingerprinting probes. I tested two of these probes on a group of isolates from Kentucky and Indiana, and also the group of isolates used by the U.K. wheat-breeding program. To my knowledge, there have been no previous studies of the diversity present in *G. zeae* in this region.

The RFLP fingerprinting probes hybridized to 26 polymorphic bands (Rep1.92 to 24, and Rep1.52 to 2), and revealed the presence of substantial genetic diversity among the Kentucky and Indiana isolates. A high degree of diversity among patterns of other neutral markers has been reported for other *G. zeae* populations in North America ( Zeller, Bowden, and Leslie, 2003, 2004; Goswami, and Kistler, 2004; Schmale et al., 2006; Gale et al., 2007; Gale et al., 2011). Isolates from the same location were more similar than isolates from different locations, and isolates with the same pattern were not recovered from more than one location, suggesting that short-distance movement dominates over long-distance migration. A similar relationship between genetic distance and geographic distance has also been reported previously (Schmale et al., 2006; Zeller et al, 2003). I recovered an average of 2.07 (+/- 0.82) genotypes from each blighted wheat head, which is comparable with the average of 1.8 genotypes / head reported by Zeller et al. (2003). The percentage of wheat heads infected by more than one genotype may provide us with a rough baseline for estimates of the frequency of out-crossing, since two parental

genotypes must be present in the same location in order for out-crossing to occur (Zeller et al, 2003).

Analysis of the U.K. wheat-breeding fungus population revealed that it was representative of the diversity across the state. There was very little redundancy, with only two of the 18 isolates apparently belonging to the same genotype. Use of the fingerprinting probes could be helpful in designing even more representative strain collections for future breeding work. A mixture of strains is normally used for inoculation. The ability to differentiate among these on the basis of the neutral RFLP markers would enable discovery of which strains are best adapted to each variety by analysis of strains colonizing symptomatic heads at the end of the season.

Among the strains isolated from Owensboro, KY, were several representing species of *Fusarium* other than *F. graminearum*. *F. acuminatum* has a broad host range, and is known as a causal agent of FHB on barley (Xue et al., 2006). On wheat it is more common as a root and crown rot (Strausbaugh et al., 2005), although it was recently found causing FHB in northern China (Wang et al., 2010). This species is reported to produce enniatins, moniliformin, and T-2 toxin (Logrieco et al., 1992). I tested the second, apparently uncharacterized *Fusarium* species for pathogenicity, but it was not aggressive on Pioneer 2555 under greenhouse conditions. The strain initially came from a severely symptomatic wheat head, so the lack of aggressiveness observed under greenhouse conditions could mean that host or environmental factors were not optimal for pathogenicity of this isolate. Alternatively, the fungus may have been present in combination with a *F. graminearum* strain, resulting in the more severe disease symptoms. More work needs to be done to characterize both of these species and to determine their distribution in Kentucky and their effect on different wheat lines in the field. Neither of these species was represented in the U.K. wheat breeders' strain collection.

In addition to being valuable tools for evaluation of genetic diversity among field isolates, the RFLP probes also proved very useful as markers for segregation analyses in crosses. Compared with CAPs, the RFLP fingerprinting probes were more efficient: the probe Rep1.92 revealed four independently-segregating polymorphic markers in a single

blot. None of the four markers were allelic to one another, nor was linkage detected between any of the markers. Because these probes hybridize to any field isolate, it should be possible to use these markers to detect and measure out-crossing in the field.

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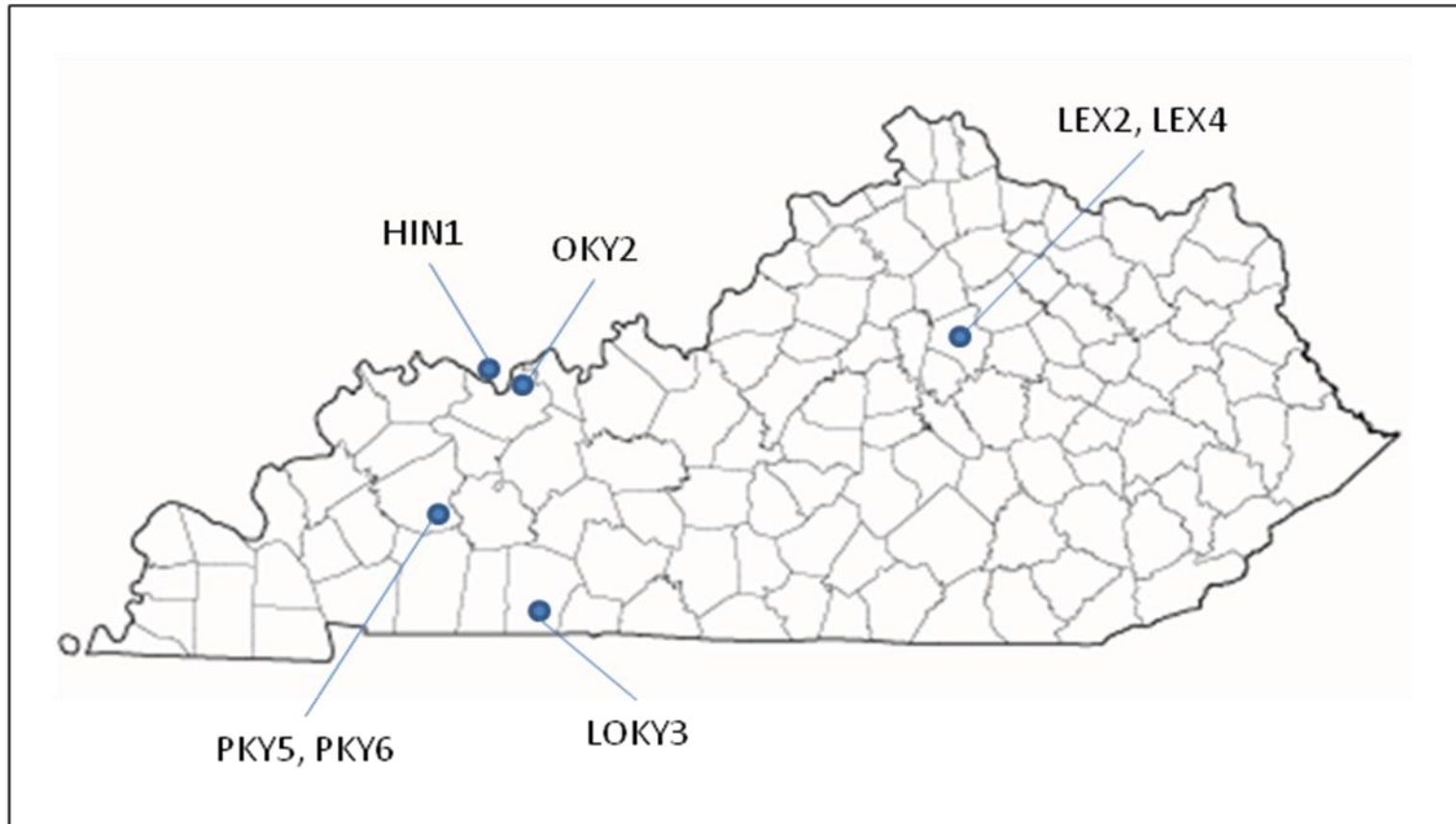
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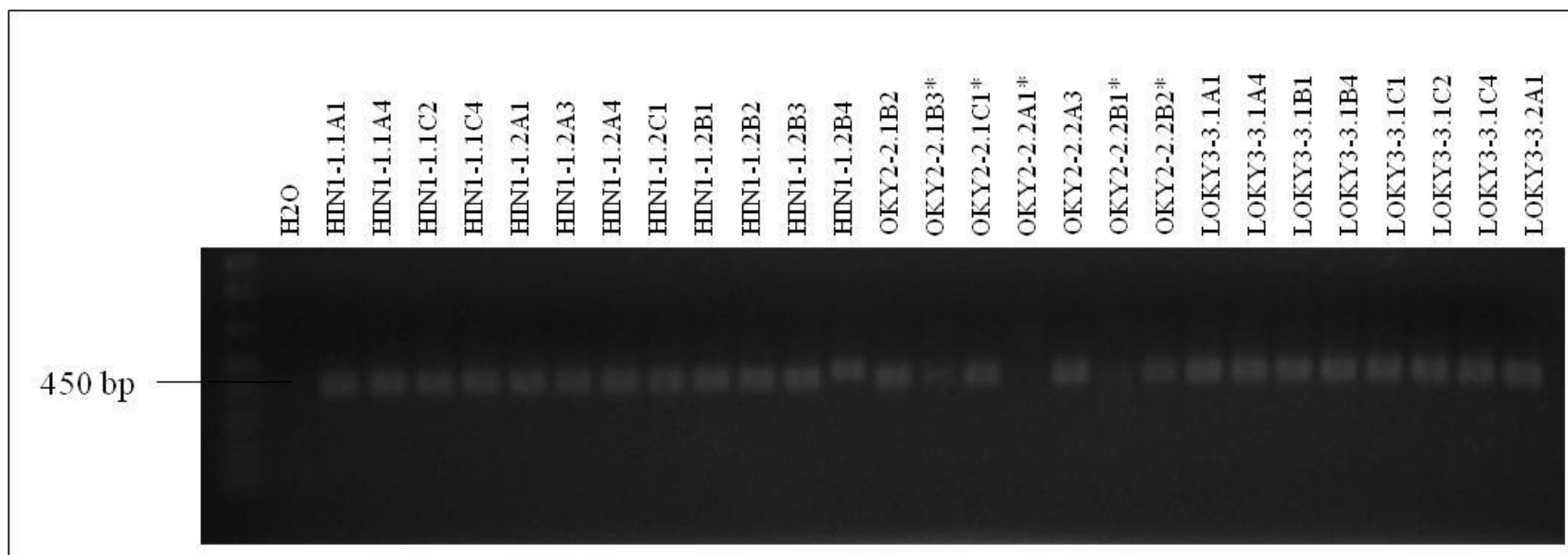
**Figure 2.1:** Origins of the fungal isolates used in the study of local *Fusarium graminearum* population structure. Map modified from one published online at <http://www.agcensus.usda.gov/Publications/images/profiles/kentucky.gif>.

**Table 2.1.** List of molecular probes used in the study of genotypic diversity of *F. graminearum* population in Kentucky. Each probe is accompanied with the information about the type of the marker they belong to, a specific restriction enzyme, primers used to amplify generate the probes, and the number of markers detected by each probe.

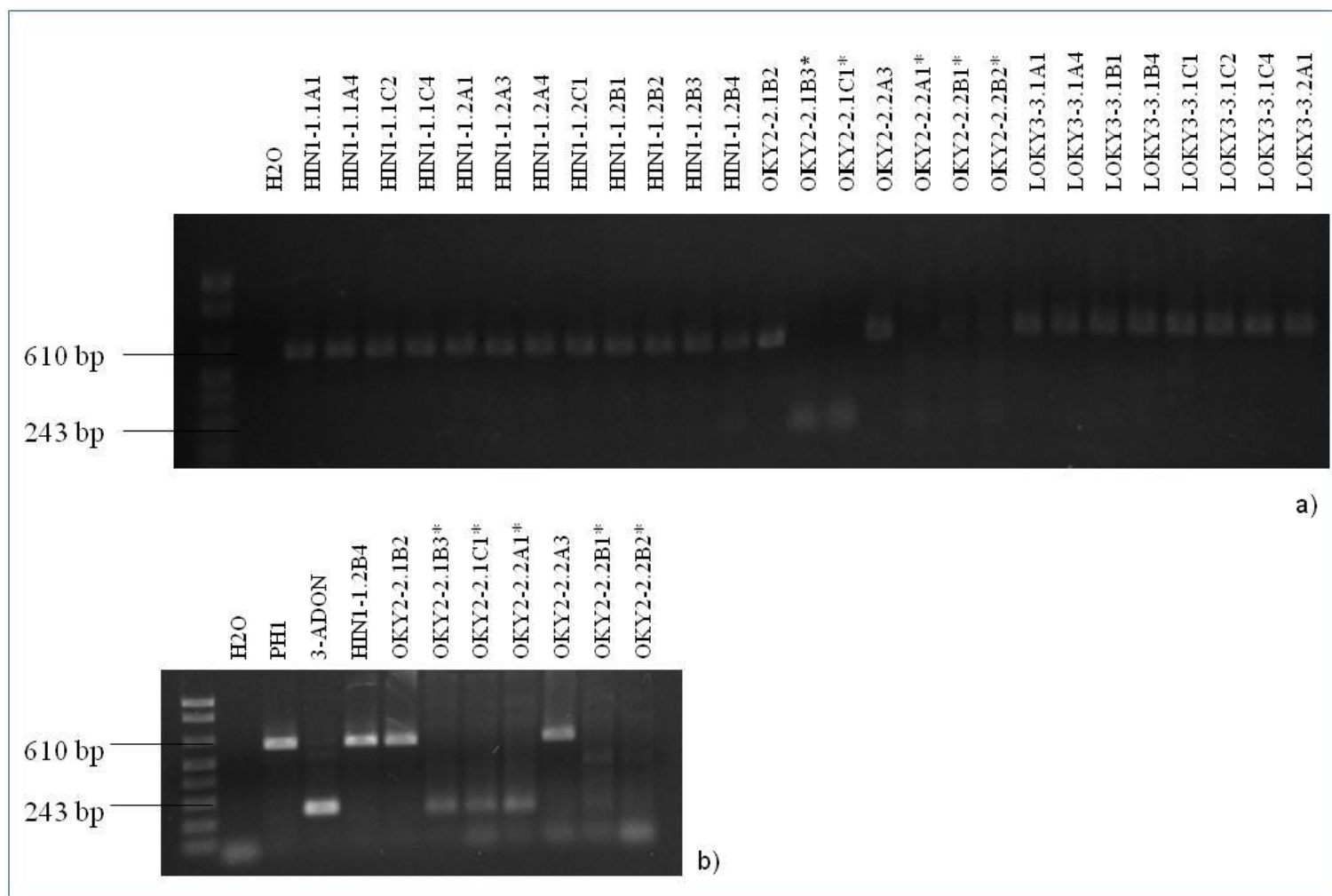
Probe	Type	RE digestion	Primer sequences (5'→3')	Number of alleles detected	Origin
REP1.52	RFLP	BsoBI	REP1.52 F (GGTTCATGAAGACATTGGTC)	2	This study
			REP1.52 R (TGATCCCGGTCTACTACATC)		This study
REP1.92	RFLP	BsoBI	REP1.92 F (GTCACCAAAGTTCGGTTCT)	24	This study
			REP1.92 R (CGGTTATGCAATCTGTTCTT)		This study
Telomere	RFLP	BsoBI	TELO F (TTAGGGTTAGGGTTAGGG)	variable	Schechtman, 1990
			TELO R (CCCTAACCCTAACCCTAA)		Schechtman, 1990
EcoRI3	CAPs	EcoRI	EcoRI3 F (GGTTCGGTGAGTCTTTAAGCCCC)	2	This study
			EcoRI3 R (CGGCTTGAGGGTTTTCGAGC)		This study
PstI2	CAPs	PstI	PstI2 F (TCGACTTGAGAGACAACCGAGCA)	2	This study
			PstI2 R (TCAAAGGGCTTGAGGAGAGTCA)		This study
Fg16	Species specific PCR marker	N/A	Fg16 F (CTCCGGATATGTTGCGTCAA)	1	Demeke et, al 2005
			Fg16 R (GGTAGGTATCCGACATGGCAA)		Demeke et, al 2005
3CON	Chemotyping probe	N/A	3CON (TGGCAAAGACTGGTTCAC)	2	Ward et al, 2002
			3D15A (ACTGACCCAAGCTGCCATC)		Ward et al, 2002
			3D3A (CGCATTGGCTAACACATG)		Ward et al, 2002

**Table 2.2:** List of fungal isolates used in the study of *F. graminearum* local Kentucky population diversity. The list includes the location strains were isolated from, number of symptomatic wheat heads per location, total number of isolates recovered, the percentage of the isolates with a unique RFLP fingerprint, and the number of symptomatic wheat heads that yielded more than one *F. graminearum* RFLP fingerprint.

Sample ID	Location	Number of wheat heads	Total number of <i>Fusarium graminearum</i> isolates	Number of other <i>Fusarium sp</i> isolates	Number of unique telomere profiles among <i>F. graminearum</i> isolates	Number of wheat heads with >1 telomere profile	Number of unique RFLP profiles among <i>F. graminearum</i> isolates	Number of wheat heads with > 1 RFLP profile
HIN1	Hatfield, IN	5	12	0	7	3	9	3
OKY2	Owensboro, KY	4	2	5	1	2	2	0
LOKY3	Logan Co. KY	4	8	0	2	0	3	0
LEX2	Lexington, KY	12	19	0	10	1	10	1
LEX4	Lexington, KY	6	18	0	13	5	15	6
PKY5	Princeton, KY	5	11	0	6	2	6	2
PKY6	Princeton, KY	5	12	0	6	2	8	2
DVS	across Kentucky	n/a	18	n/a	14	n/a	17	n/a

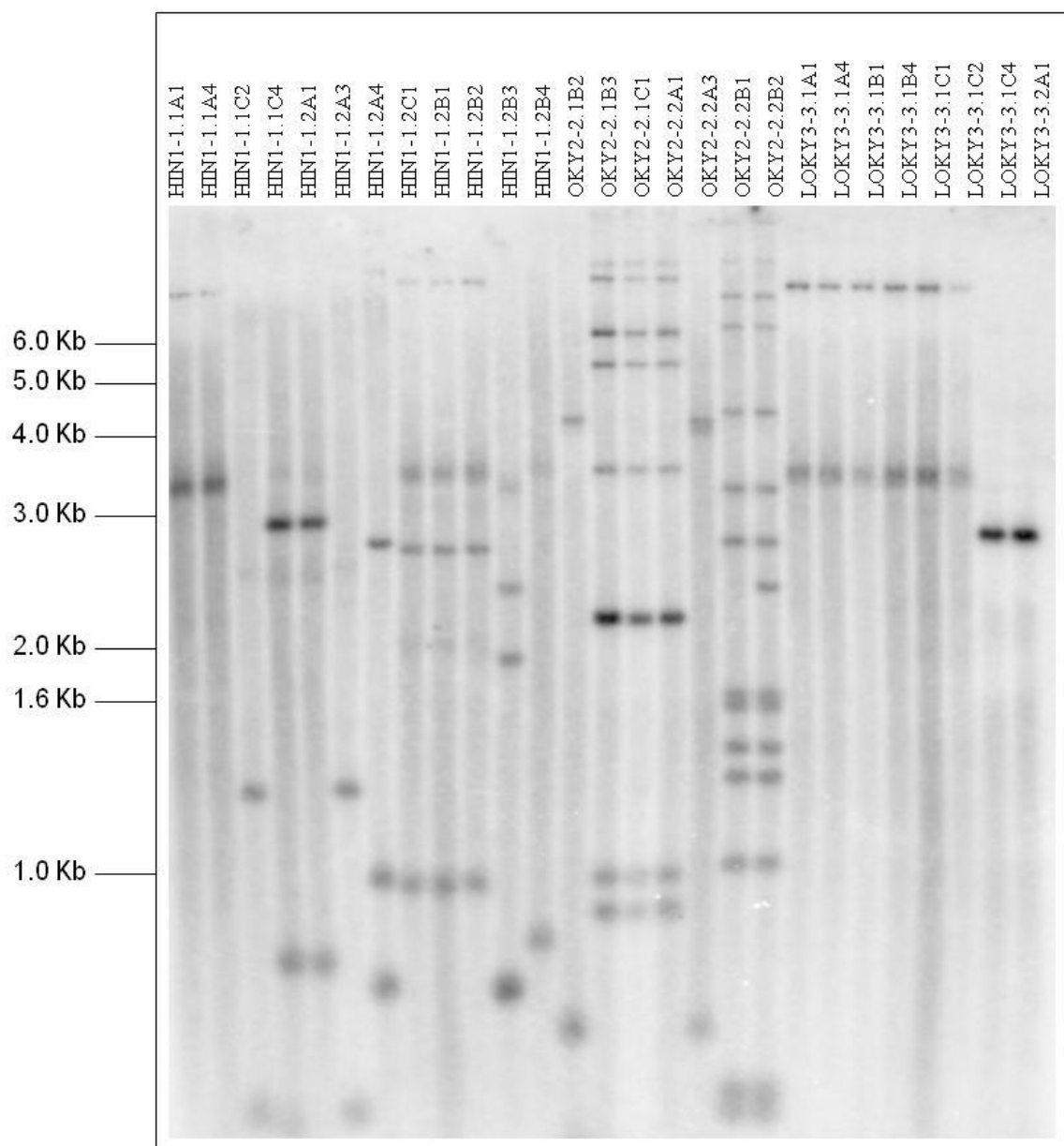


**Figure 2.2.** Analysis of a subset of fungal isolates (HIN1, OKY2, and LOKY3) using the *Fusarium graminearum* species-specific marker Fg16. (approximate amplicon size: 450bp) (Demeke et al, 2005). Asterisks mark isolate that did not amplify the expected product with the primers.

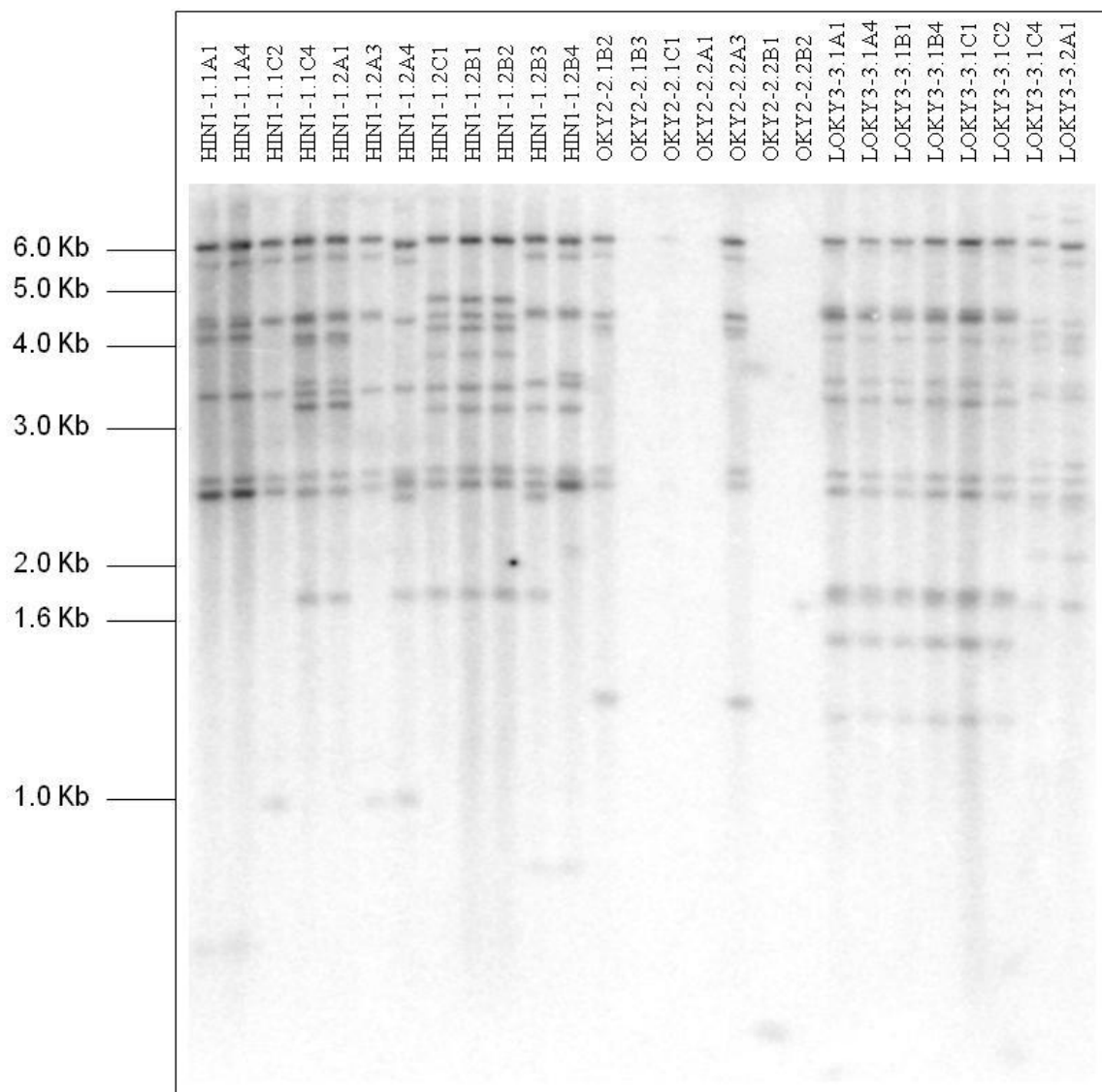


**Figure 2.3.** An example of chemotype characterization of a subset of fungal isolates using primer set 3CON (Ward et al, 2002). The expected amplicon size specific for the 15-ADON chemotype is 610 bp, and for the 3-ADON chemotype it is 243 bp. Asterisks mark isolates that did not amplify the expected products with the primers.

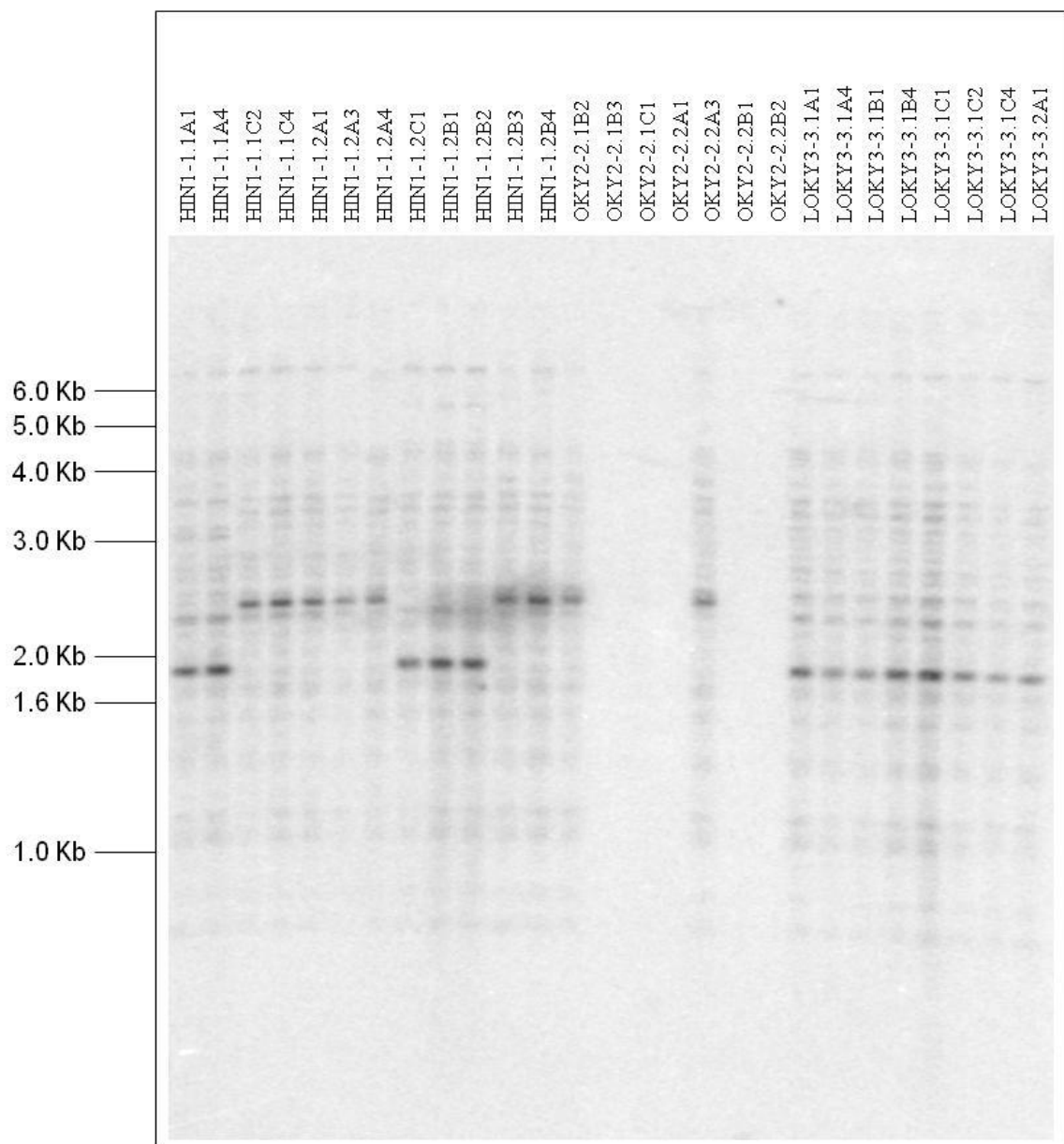




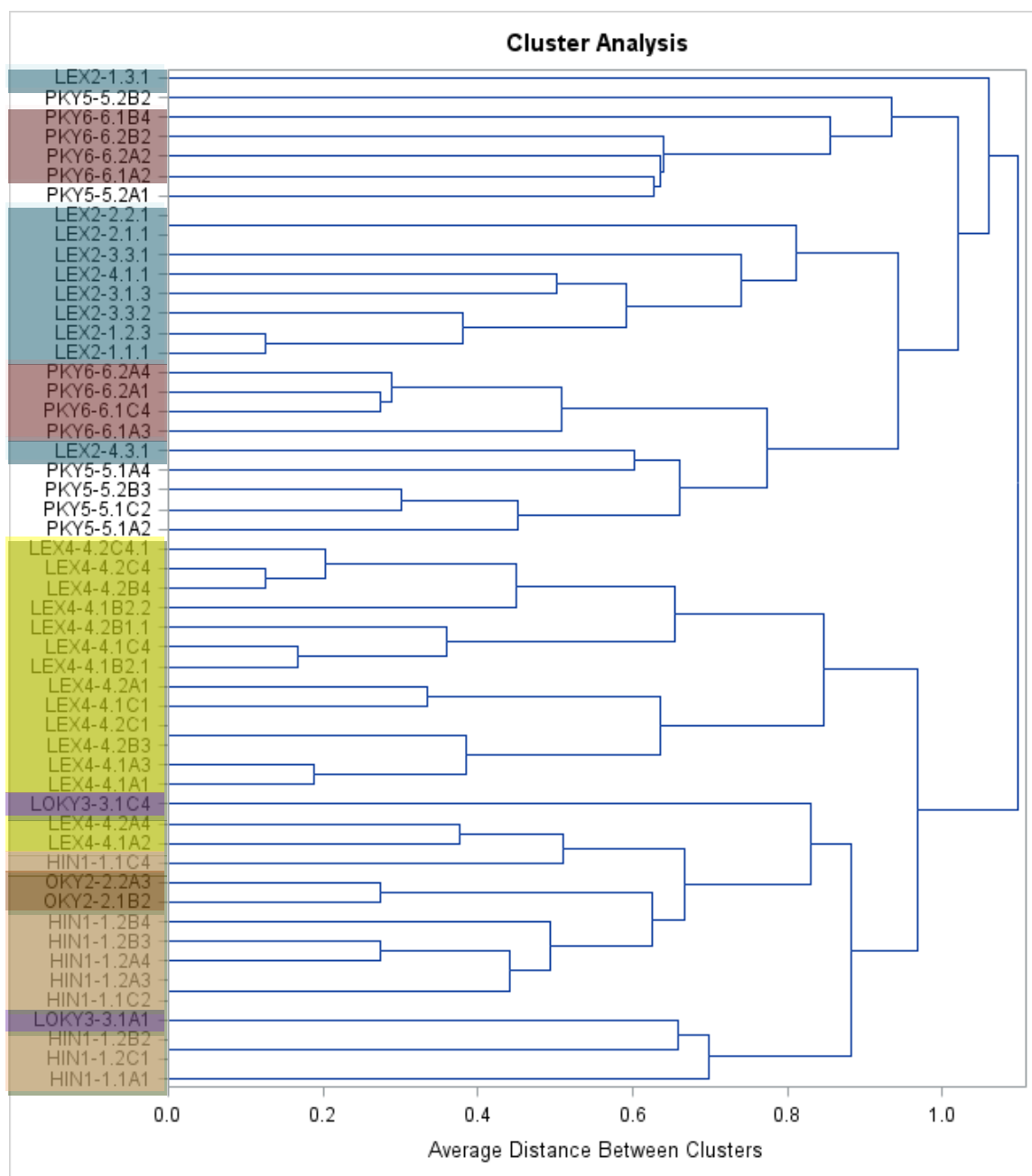
**Figure 2.4:** An example of a Southern blot analysis of the *BsoBI* digest of fungal isolates from Hartfield, IN; Owensboro, KY and Logan Co., KY. The digest was probed with a (TTAGGG)<sub>n</sub> telomere specific probe.



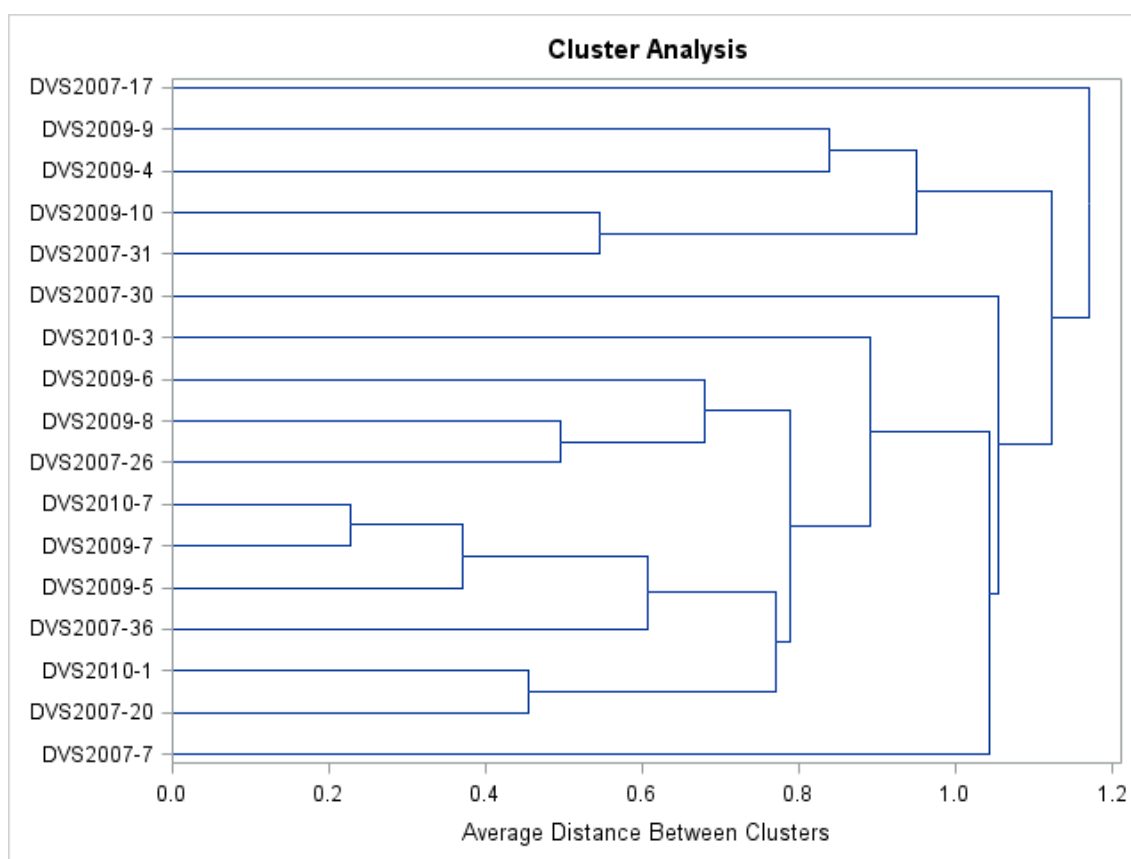
**Figure 2.5:** An example of Southern blot analysis of the *BsoBI* digest of the sets of fungal isolates from Hartfield, IN; Owensboro, KY and Logan Co., KY probed with Rep 1.92 probe.



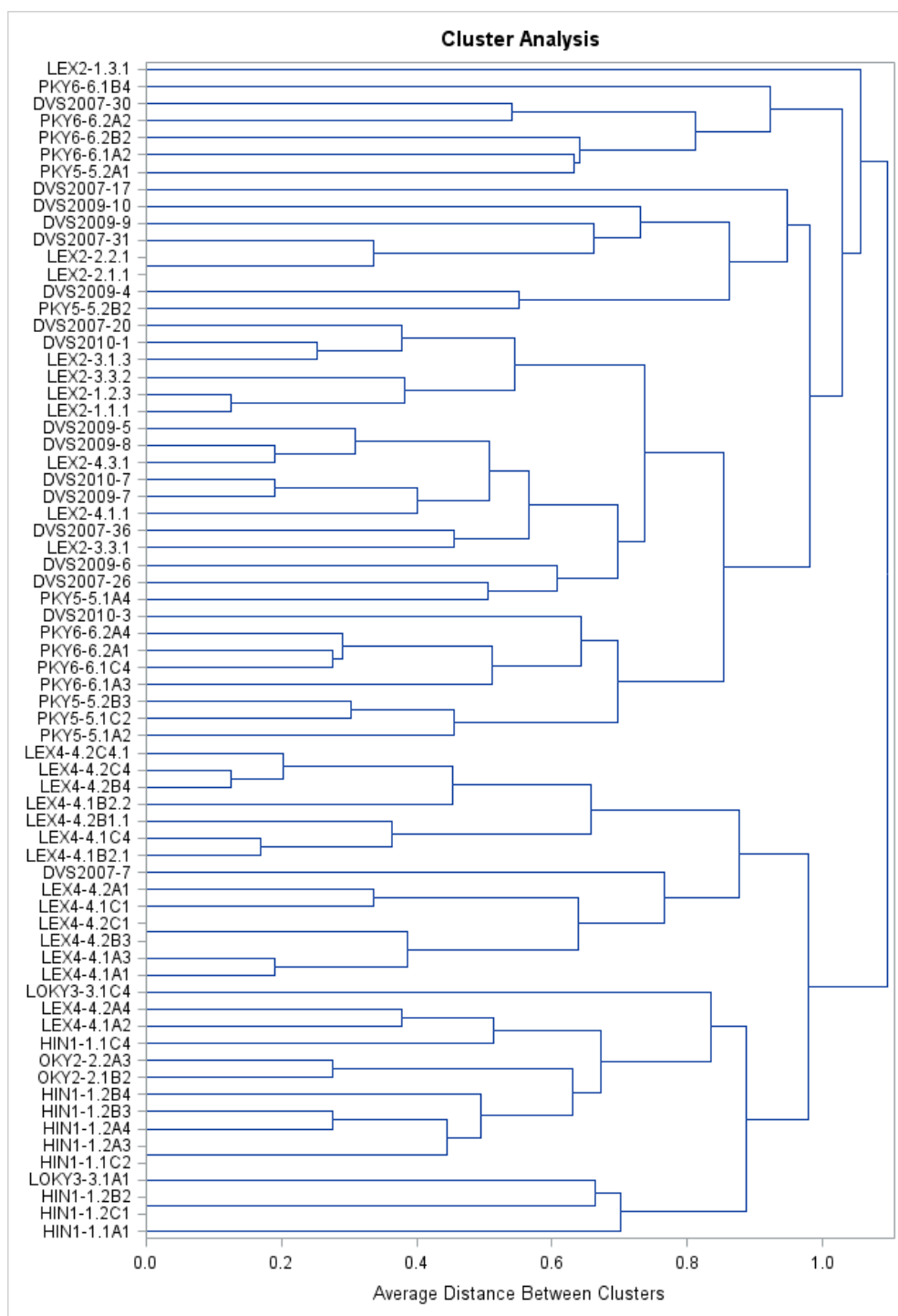
**Figure 2.6:** An example of Southern blot analysis of the *Bso*BI digest of the sets of fungal isolates from Hartfield, IN; Owensboro, KY and Logan Co., KY probed with RFLP probe Rep1.52.



**Figure 2.7:** UPGMA "dendrogram" ( nor ) of *Fusarium graminearum* genotypes representing isolates collected across Kentucky and Indiana. The samples were collected from two fields in Lexington KY [LEX2 (blue) and LEX4 (yellow)]; two fields in Princeton, KY [PKY5 (white), and PKY6 (red)]; one field in Owensboro, KY [OKY2 (brown)]; one field in Logan Co., KY [LOKY3 (purple)], and one field in Hartfield, IN [HIN1 (tan)]. The UPGMA dendrogram was constructed based on Jaccard similarity matrix among RFLP profiles from *Bso*BI DNA digests probed with RFLP probe Rep 1.92. Scale bar indicates Jaccard similarity values.



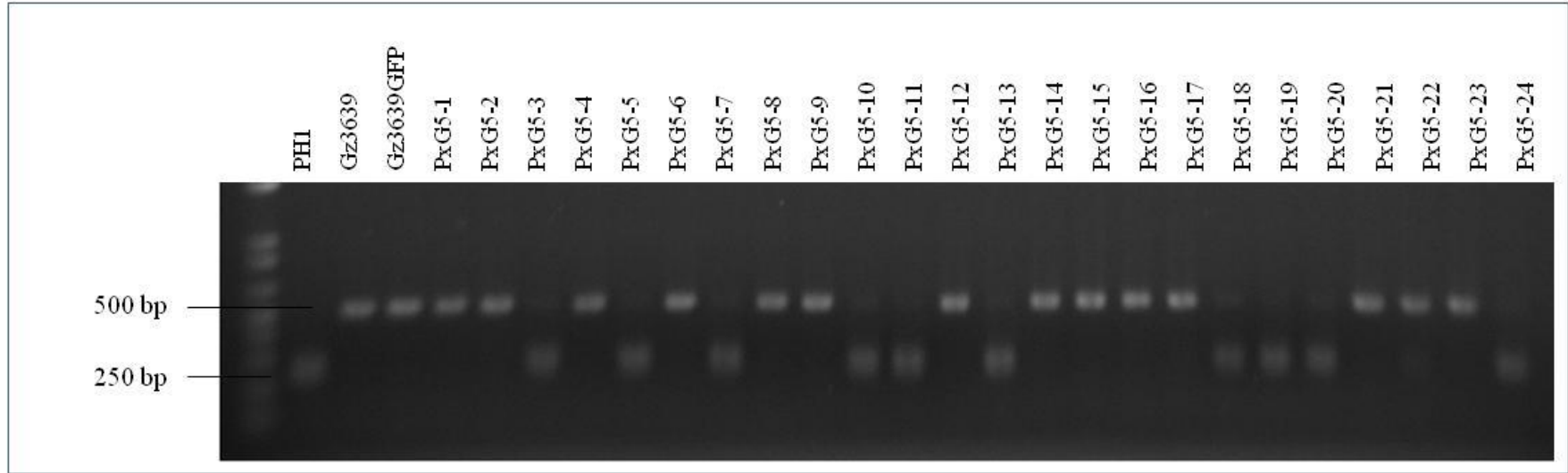
**Figure 2.8:** UPGMA " dendrogram" ( nor ) of genotypes of *Fusarium graminearum* isolates used by the U.K. wheat breeding group. The samples were collected from across Kentucky between 2007-2010. The UPGMA dendrogram was constructed based on the Jaccard similarity matrix among RFLP profiles from *Bso*BI DNA digests probed with repetitive probe Rep 1.92. Scale bar indicates Jaccard similarity values.



**Figure 2.9:** UPGMA " dendrogram" ( nor ) of all the Kentucky and Indiana *Fusarium graminearum* strains used in the study of local *F. graminearum* population diversity. The UPGMA dendrogram was constructed based on Jaccard similarity matrix among RFLP profiles from *Bso*BI DNA digests probed with the RFLP probe Rep 1.92. Scale bar indicates Jaccard similarity values.

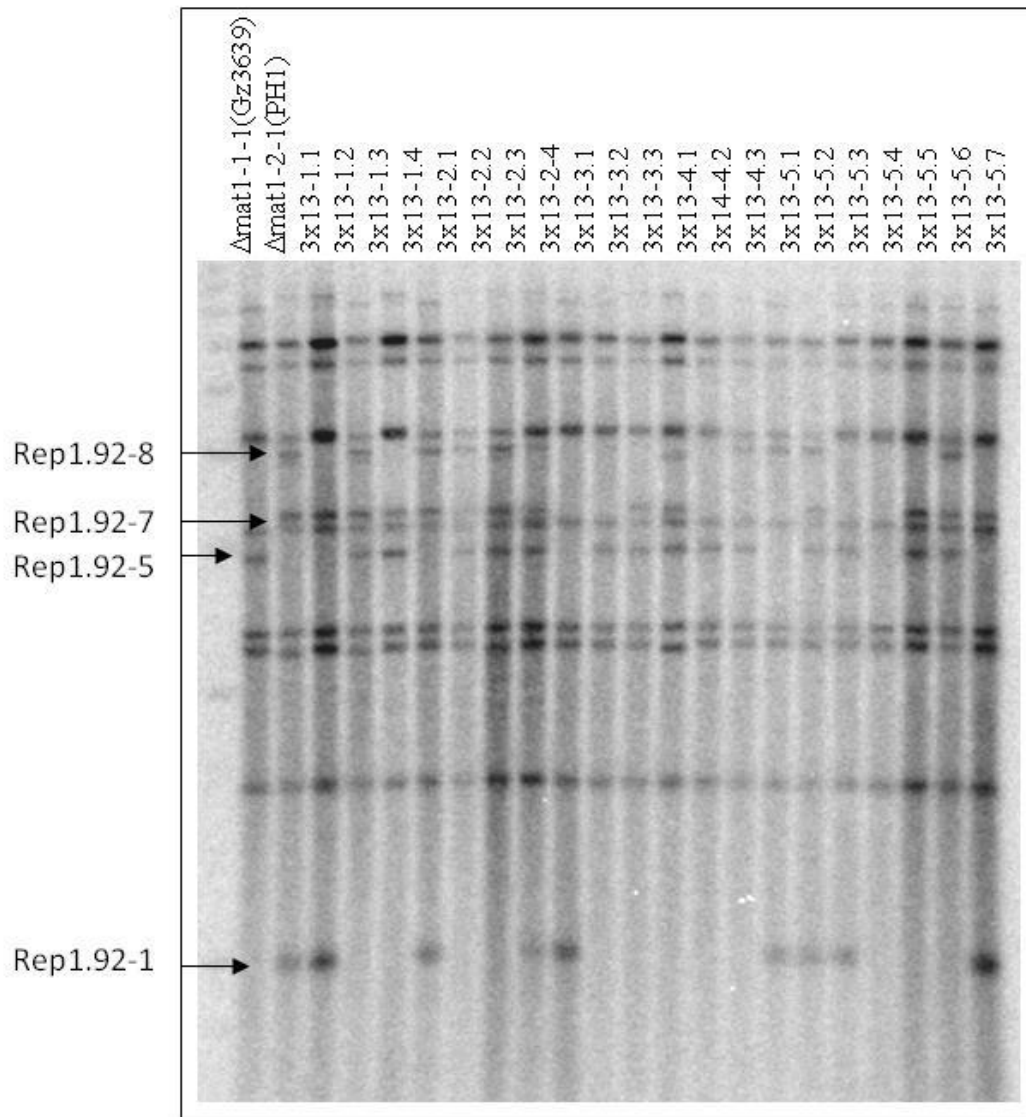


**Figure 2.10:** Comparison of macroconidial morphology and FHB aggressiveness (7 dpi) of *Fusarium graminearum* strain PH-1 (a and c), and an unidentified *Fusarium* sp. OKY2-2.2B1 (b and d), which was isolated from a FHB symptomatic wheat head collected in Owensboro, KY. The difference between PH-1 and the unidentified *Fusarium* sp. in apical cell morphology is shown by black arrows; the point of inoculation on wheat heads is indicated by white arrows.



**Figure 2.11.** A representative example of the SNP/CAPs analysis of a set of progeny from a PH-1 x Gz3639 cross. The genomic sequence containing the single nucleotide polymorphism (SNP) marker was amplified from both parental strains and from the progeny strains with the *PstI*2 PCR primer set (Table 1) and digested with *PstI*. The digests were separated on a 0.8% agarose gel and visualized under UV light. Segregation ratio of PH-1: Gz3639 allele =(10:15);  $\chi^2= 0.66$ ; P-value = 0.99.





**Figure 2.12:** Example of the use of Rep1.92 probe to detect recombination among polymorphic RFLP markers in the progeny of a cross between  $\Delta\text{mat1-1-1}$  (Gz3639) and  $\Delta\text{mat1-2-1}$ (PH-1). Bands that are polymorphic between the two parent strains are indicated by the arrows.

**Table 2.3:** Segregation analyses for the repetitive RFLP molecular markers among F1 progeny from a cross between  $\Delta\text{mat1-1-1}$  (Gz3639) and  $\Delta\text{mat1-2-1(PH-1)}$ .

Marker	Segregation		Df	$\chi^2$	P-value
	Expected (1:1)	Observed (1:1)			
Rep1.92-1	10:10	8:12	19	0.4	1
Rep1.92-5	10:10	14:6	19	1.6	0.9999
Rep1.92-7	10:10	8:12	19	0.4	1
Rep1.92-8	10:10	9:11	19	1	0.9999
Marker combinations	Expected (1:1:1:1)	Observed (1:1:1:1)	Df	$\chi^2$	P-value
Rep1.92-1 Rep1.92-5	10:10:10:10	8:12:14:6	39	4	1
Rep1.92-1 Rep1.92-7	10:10:10:10	8:12:8:12	39	1.6	1
Rep1.92-1 Rep1.92-8	10:10:10:10	8:12:9:11	39	1	0.9999
Rep1.92-5 Rep1.92-7	10:10:10:10	14:6:8:12	39	4	1
Rep1.92-5 Rep1.92-8	10:10:10:10	14:6:9:11	39	3.4	0.9999
Rep1.92-7 Rep1.92-8	10:10:10:10	8:12:9:11	39	1	0.9999

## CHAPTER 3

### **Crossing two genetically and phenotypically similar *Gibberella zeae* strains produces transgressive progeny with increased levels of aggressiveness and toxigenicity**

#### **3.1 Introduction**

Fusarium head blight (FHB), aka scab, is one of the most important diseases of wheat and barley in North America and worldwide (Goswami, and Kistler, 2004; Miedaner, Cumagun, and Chakraborty, 2008; Ramirez et al., 2007). When environmental conditions are favorable for development of epidemics, yield losses can be as much as 50% (Cowger and Sutton, 2005). Losses are caused by decreased seed number and weight, and by reduced grain quality, mainly due to contamination with mycotoxins (Desjardins, 2006; Leslie and Summerell, 2006). Two major classes of mycotoxins are produced in the blighted heads: the non-estrogenic trichothecenes deoxynivalenol (DON) and nivalenol (NIV); and the mycoestrogens zearalenone and zearalenol (Desjardins, 2006). Because of its extreme negative effect on animal and human health, DON in particular has become a major concern. The amount of DON in grain and finished products intended for human consumption cannot exceed 1 ppm (Council for Agricultural Science and Technology, 2003). U.S. wheat and barley farmers lost nearly 2.5 billion dollars as a direct result of FHB and DON contamination during the 1990s (Njanje, Bangsund, and Leistritz, 2002).

*Gibberella zeae* (anamorph *Fusarium graminearum*) is the primary causal agent of FHB in North America (Trail, 2009). *G. zeae* is a homothallic ascomycete with the ability to out-cross (Bowden and Leslie, 1999). Ascospores produced on crop debris serve as the primary inoculum for FHB epidemics in the spring (Doohan, Brennan, and Cooke, 2003; Osborne and Stein, 2007; Trail, 2009). Co-infection by multiple strains could result in out-crossing and recombination among pathogenicity alleles, which could in theory produce transgressive progeny that are more aggressive or more toxigenic than either parent. An outcrossing frequency of up to 35% was reported on carrot agar media under laboratory conditions (Bowden and Leslie, 1999). There are no data to indicate the rate of out-crossing in the field. However, the high level of genetic diversity found among

field isolates of *G. zeae* suggests that sexual recombination does occur (Gale et al., 2002; Zeller, Bowden, and Leslie, 2004).

Based on the DNA sequences of 11 genes, *G. zeae* was proposed to consist of nine genetic lineages (aka. phylogenetic species) (O'Donnell et al., 2004). The results of additional phylogenetic analysis have established *F. graminearum* as a species complex comprised of thirteen distinct phylogenetic species (Starkey et al., 2007).

The majority of the strains causing FHB in North America belong to lineage 7 (O'Donnell et al., 2004). The complete genomes of two representative lineage 7 *G. zeae* strains have been sequenced. Strain PH-1, isolated in Michigan, was one of the parents in an intraspecific cross to generate a genetic map of *G. zeae* (Gale et al., 2005). The genome sequence of PH-1 became publicly available as a result of a collaboration between the Broad Institute, the Massachusetts Institute of Technology, and the International *G. zeae* Genomics Consortium (IGGR) (Cuomo et al., 2007). The genome assembly is 36.4 Mb in length with ~11x coverage, and contains about 13,000 annotated genes. Shortly after the PH-1 genome sequence became publicly available, Syngenta (Wilmington, DE) released a genome sequence for a second strain, Gz3639 (Gale et al., 2005). This strain, isolated in Kansas, has been utilized for much of the mycotoxin research and has also been used as one of the parents in an interspecific cross with a Japanese strain (a member of lineage 6) to generate another genetic map (Jurgenson et al., 2002). The genome sequence of Gz3639 consists of 24,000 shotgun reads, representing 0.4x genome coverage. Alignment of these two genome sequences generated a list of 10,500 single-nucleotide polymorphism (SNP) markers (Cuomo et al., 2007). The objective of my research was to test the hypothesis that an intraspecific cross between these two well-characterized lineage 7 *F. graminearum* strains would generate transgressive progeny that were more aggressive or more toxigenic than the parent strains.

### 3.2 Materials and Methods

**Fungal culture conditions:** All strains were grown at 23° C with constant light (Sylvania F032/741/ECO). Strains were single-spored and stored on silica gel at -20° C or -80° C (Tuite, 1969, after Perkins, 1962). Strains were never subcultured more than once. Spore production was evaluated on two different types of media. Production of asexual spores (macroconidia) was assessed after 7 days of growth on mungbean agar (Bai and Shaner, 1996). Production of asexual and sexual spores (ascospores) was measured after 21 days of growth on carrot agar media (Klittich and Leslie, 1988). In each case, 2 ml of sterile water was applied to the surface of the Petri plate and the spores were released by rubbing with a sterile plastic micro-pestle. The spore suspension was filtered through glass wool to remove mycelial fragments. Spores were counted by using a hemocytometer. For use as inoculum, spores were washed once in sterile water and the concentration was adjusted as necessary.

**Sexual Crosses:** *G. zeae* strains were crossed using the mycelial plug method described by Bowden and Leslie (1999). Five-mm plugs of mycelia of each parental strain, taken from the edges of actively growing cultures on potato dextrose agar (PDA), were placed on opposite halves of a 60 mm Petri plate. After four days of incubation at 23° C, perithecial production was induced by applying 500-1000 µl of 2.5% Tween 60 to the surface of each plate, and gently rubbing the aerial mycelium with a sterile glass rod to flatten it. Following induction, the plates were incubated at 23° C with constant fluorescent light until perithecial maturation. The mature perithecia extruded cirrhi that contained hundreds of ascospores 2-3 weeks after induction. A sterile glass needle was used to pick up individual cirrhi, which were then dispersed in water and plated on 2% water agar. After 10-14 hours of growth on water agar, isolates arising from single ascospores were transferred to individual 6 mm Petri plates. No more than 25 single-ascospore isolates per perithecium were used for subsequent genetic analyses.

**GFP screening method:** To facilitate the identification of perithecia that resulted from out-crossing, one of the parental strains (Gz3639) was transformed to express green fluorescent protein (GFP). The half of the crossing plate containing the transformant fluoresced green, whereas the half containing the wild-type strain was dark. Occasionally, green-fluorescent cirrhi were seen on the dark half, and these were assumed to have arisen from out-crossing. The fluorescent cirrhi were recovered and single-ascospore progeny were isolated as above. Twenty-three or 24 single-spore progeny from each of four fluorescent cirrhi were subsequently tested by using single-nucleotide polymorphism (SNP) markers to confirm that they were the product of outcrossing.

**DNA extraction:** To isolate fungal DNA, five ml aliquots of YEPD media (20 g dextrose, 10 g Bacto® peptone, 3 g yeast extract per L) in 50 ml glass tubes were inoculated with 8-mm plugs of an actively growing culture and incubated for 5-7 days at room temperature with agitation (250 rpm). The mycelial mats were harvested by filtration, frozen at -80°C for one hour, and lyophilized in a freeze-drier. Lyophilized tissue was pulverized with steel beads in 96-well plates in a GenoGrinder® 2000 (500 strokes/s) for 1 minute. Pulverized tissue was suspended in lysing buffer (5 M NaCl, 1M Tris-HCl, pH7.5; 0.5M EDTA, 10% SDS) for 30 minutes at 65°C. The solution was subsequently extracted with 1 volume of phenol:chloroform:isoamyl alcohol (25:24:1) for an additional 30 min at 65°C. The resulting slurry was centrifuged for 20 min at 13,000 rpm to separate the phases. The aqueous phase (500-600 µl) was transferred into a clean Eppendorf tube and precipitated with 0.6 volume of isopropanol. The samples were centrifuged for 10 min at 13,000 rpm to pellet the DNA. The pellet was washed twice with 70% ethanol. After the ethanol washes, the DNA pellet was dried for 10 minutes in a transfer hood, then dissolved in 100 µl TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0) + 10 µg RNaseA at room temperature.

**Preparation of fungal protoplasts:** Protoplast isolation and transformation protocols were modified from methods used for transformation of *Aspergillus parasiticus* (Skory et al. 1990; F. Trail, personal communication). Spores were harvested from 7 day-old cultures grown on mungbean agar. The spores were washed and the concentration was adjusted to  $1 \times 10^6$  spores/ml. Two ml of the spore suspension was used to inoculate 100 ml of YEPD. The cultures were incubated for 10-14 hours at 25°C with constant agitation (175 rpm). The mycelium was harvested by filtration, washed with sterile water, and then suspended in 20 ml of protoplasting buffer [500 mg driselase (Sigma Chemical Co., St. Louis), and 100 mg lysing enzyme from *Trichoderma harzianum* L-1412 (Sigma Chemical Co., St. Louis), in 1.2 M KCl]. The mixture was incubated for 45-90 minutes at 37°C with gentle agitation (50-80 rpm). When the majority of hyphae had released protoplasts, the solution was filtered through a 30 µm Nitex nylon membrane into 50 ml centrifuge tubes. The protoplasts were collected by centrifugation (3000 rpm) at room temperature for 5 minutes, washed twice in STC buffer (1.2 M sorbitol, 10 mM tris-HCl, pH 8.0, 50 mM CaCl<sub>2</sub>), and then resuspended in the same buffer at a concentration of  $1 \times 10^8$  protoplasts/ml.

**Transformation of *Fusarium graminearum*:** For each transformation, 100 µl of a fresh protoplast suspension ( $1 \times 10^8$  protoplast/ml) was mixed with 100 µl STC, 50 µl freshly-prepared and filter-sterilized 30% PEG solution [30% w/v 8000 polyethylene glycol (Sigma, P2139) in buffer (10 mM tris-HCl, pH 8.0; 50 mM CaCl<sub>2</sub>)], and 10 µl of *EcoRI*-linearized pCT75 DNA (1-3 µg) (Lorang et al., 2001). The transformation reaction was incubated at room temperature for 20 minutes, followed by addition of 2 ml 30% PEG Solution and an additional 5-minute incubation. Four ml of STC was added and mixed gently by inversion. The entire transformation reaction was then poured into 250 ml of cooled regeneration medium (RM) [1.0 g yeast extract, 1.0 g N-Z-Amine AS (N4517 Sigma), 7.44 g agar, and 271 g sucrose per L] and aliquoted into 100 mm Petri plates. Transformed protoplasts were regenerated for 12-15 hours and then overlaid with 15 ml of regeneration medium amended with 150 µg/ml hygromycin B. Transformants usually

appeared within 4-7 days, and were transferred to PDA medium containing 450 µg/ml hygromycin B.

### **Generation of molecular probes**

**CAPS markers:** Cleaved Amplified Polymorphic Sequences (CAPS) markers were generated by using a list of single-nucleotide polymorphisms (SNPs) differentiating PH-1 and Gz3639 (Cuomo et al., 2007). The list of SNPs was downloaded from ([http://www.broadinstitute.org/annotation/genome/fusarium\\_graminearum/MultiDownloads.html](http://www.broadinstitute.org/annotation/genome/fusarium_graminearum/MultiDownloads.html)). Four unlinked SNPs that spanned *EcoRI* and *PstI* restriction sites were identified, and a ~500 bp segment spanning each selected SNP site was amplified by using primer sets that were designed manually for each region (Table 3.1). PCR amplification reactions contained Phusion polymerase (Finnzymes F-530S) and other components according to the manufacturer's instructions. The thermocycling protocol consisted of initial denaturation for 1 min. 30 sec. at 94° C; followed by 35 cycles of 30 sec denaturation at 94° C, 20 sec annealing at 68° C, and 45 sec extension at 72° C; and one extension cycle for 7 min. at 72° C. Three µL of each PCR amplicon was used for each restriction reaction. Restriction reactions used Invitrogen restriction enzymes *EcoRI* (Invitrogen Cat #15202-015) and *PstI* (Invitrogen Cat. # 15215-015) according to the manufacturer's instructions. Restriction reactions were separated on a 1% agarose gel for analysis.

**Telomere marker:** A telomere probe was generated by using two oligomer primers (Table 3.1). The probe was synthesized by performing PCR without a template, resulting in generation and amplification of primer dimers and very large increases in product size in the later cycles (Schechtman, 1990). Phusion polymerase, which has proofreading ability, was used for the PCR. Twenty pmol of each primer was added to each PCR reaction mix with the manufacturer's recommended buffer. The thermocycling protocol consisted of 35 cycles of denaturation for 30 sec. at 94° C, annealing for 30 sec. at 50° C and extension for 60 sec at 72° C; followed by one 2-min. extension at 72° C. PCR



products were separated on 1% agarose gel. DNA with a size range of between 1.5 and 2 Kb was excised and purified with a gel extraction kit (QIAquick®, Quiagen cat.#28704). The purified product (50-80 ng) was labeled using the Prime-a-gene labeling system (Promega, cat.#U1100) and used for Southern hybridization analysis (Southern, 1975).

**Repetitive RFLP markers:** Repetitive sequences were identified by blasting the PH-1 genome sequence against itself. From among the list of repetitive sequences, two (REP1.52 and REP1.92) were selected and primers were designed to amplify them (Table 3.1). The PCR reactions were performed with Pfu polymerase according to the manufacturer's recommendations. The thermocycling protocol consisted of initial denaturation for 3 min. at 94° C, followed by 40 cycles of denaturation for 30 sec. at 94° C, 20 sec. annealing at 55° C, 45 sec. extension at 72° C, and a final extension for 7 min. at 72° C. The PCR products were gel-purified and sequenced. Confirmed amplicons (50-80 ng) were labeled with <sup>32</sup>P using the Prime-a-gene labeling kit, and used as probes for Southern hybridization analysis (Southern, 1975).

**FHB pathogenicity assay:** Three varieties of SRWW, including Pioneer 2555 (susceptible), Pioneer 25R18 (type II resistance), and "Truman" (native quantitative resistance, moderately resistant), were used for this study. Wheat seeds were planted in a mixture of topsoil (Maury silt loam) and PromixBX growth substrate (3:2) in a 72-cell format at the rate of three seeds per cell. The seeds were lightly covered with a moist layer of soil mixture. Seeds were germinated in the greenhouse for 3-5 days at ambient temperature of 25 °C, and 12 h photoperiod. Shortly after germination and seedling emergence, seedlings were treated with the systemic fungicide NOVA™ 40W (Dow AgroSciences Canada Inc.) at the recommended rate for powdery mildew, and transferred to a cold room (~4 °C) with constant fluorescent light for 8 weeks. At the end of the vernalization period, the seedlings were transplanted in cone containers filled with a mixture of topsoil (Maury silt loam) and PromixBX (3:2). Transplanted wheat plants were grown in greenhouse with a 14/10 photoperiod, provided by "Hortilux" LU430S/HTL/EN high pressure sodium lights, and ambient temperatures between 25-

28°C. Transplants were fertilized with 150 ppm of N:P:K (20:10:20) fertilizer formulation every 14 days starting two weeks after transplanting, with the last fertilization at heading. Flowering typically occurred after 3-4 weeks. At early- to mid-anthesis, a single centrally positioned floret on the primary flowering stem of each plant was inoculated with 10 µl of a spore suspension as described by (Miedaner, Moldovan, and Ittu, 2003). Symptom severity was recorded at seven and ten days post-inoculation, as the number of FHB symptomatic spikelets per inoculated spike.

**DON production in planta:** Inoculated plants were grown in the greenhouse to physiological maturity, then whole heads were harvested, threshed, and the seeds sent for GC-MS mycotoxin analysis by Dr. Yanhong Dong at the USDA ARS Cereal Disease Lab, St. Paul, MN (Fuentes et al, 2005; Mirocha et al, 1998).

**DON production in media:** For estimation of DON production in liquid media, a modified protocol of Miller et al, (1983), was used. Briefly, 5 ml of 2% GYEP (2% D-glucose, 0.1% yeast extract, 0.1% peptone) was inoculated with 1 ml of spore suspension at a concentration of  $5 \times 10^4$  spores/ml. The liquid cultures were incubated for 14 days in the dark at ambient temperature with constant agitation (200 rpm). After the 14-day incubation period, 1 ml of the liquid culture was filtered through a glass wool filter. A 500-µL subsample was freeze-dried and sent for GC-MS mycotoxin analysis by Dr. Yanhong Dong at the USDA ARS Cereal Disease Lab, St. Paul, MN (Fuentes et al, 2005; Mirocha et al, 1998).

### 3.3 Results

**Comparison of the Parental Strains:** The two parental strains, PH-1 and Gz3639, appeared similar on PDA, although PH-1 secreted more red pigment into the media, and it produced more aerial mycelium than Gz3639 (Figure 3.1a). PH-1 produced more macroconidia than Gz3639 on CMC medium, but fewer on PDA. There was no difference in macroconidial production by the two strains on MBA (Figure 3.1b). PH-1 grew at a faster rate than Gz3639 on all three types of media (Figure 3.1 c). PH-1 produced more perithecia and more ascospores on carrot agar than Gz3639 (Figure 3.2).

When inoculated on the three SRWW varieties in the greenhouse, the two strains behaved similarly. On the susceptible variety Pioneer 2555, both strains produced severe FHB symptoms (Figure 3.3Ac). There were no significant differences in aggressiveness between the strains at three different inoculum concentrations (Figure 3.3Aa). Gz3639 induced slightly faster symptom development over time compared to PH-1, but the difference was not statistically significant (Figure 3.3Ab). Neither PH-1 nor Gz3639 were able to break the resistance present in varieties Pioneer 25R18 (Figure 3.3B) and Truman (Figure 3.3C).

Gz3639 produced more DON than PH-1 in liquid media (2% GYEPD), and *in planta* on the susceptible variety Pioneer 2555 (Figure 3.4).

**Analysis of Progeny of a Cross Between PH-1 and Gz3639-GFP:** Four perithecia extruding green fluorescent cirrhi were chosen at random from the dark PH-1 side of the crossing plate, and 23 or 24 single-spored progeny were collected from each of the cirrhi (Figure 3.5). These progeny of the cross between PH-1 and Gz3639-GFP (PxG) were screened for segregation of two unlinked CAPS markers. The two markers segregated among the progeny in the expected 1:1 ratio, and recombination among the markers occurred in the expected 1:1:1:1 ratio, confirming that all four of the perithecia were the result of out-crossing (Table 3.2).

The progeny were morphologically highly variable on PDA (Figure 3.6). Twelve out of 95 strains were much more fertile than either parent, producing abundant perithecia on

this normally non-inductive medium (Figure 3.6a). Seventeen out of 95 progeny produced unusually high quantities of orange and yellow pigments in the aerial mycelia and in the media (Figure 3.6b). Six out of 95 strains produced a thick mycelial mat and very few macroconidia (Figure 3.6c).

The progeny displayed a broad range of asexual fecundity on MBA. The asexual frequency distribution was shifted in the direction of reduced spore production, but there were also a few progeny strains that produced very large numbers of macroconidia, more than 10 times the number of spores produced by either parent (Figure 3.7).

All 95 of the progeny were inoculated onto the three varieties of SRWW. None of the progeny were able to break the resistance present in Pioneer 25R18 (Figure 3.8a), or Truman (Figure 3.8b). On the susceptible variety Pioneer 2555, the progeny displayed a broad range of aggressiveness, and several of the progeny were significantly more aggressive than either parent (Figures 3.9 and 3.10). The four most aggressive and the four least aggressive progeny strains were chosen for further analysis.

**Further characterization of transgressive progeny:** Among the original 95 progeny, there was a (non-significant) skew in the chi-square ratios of the CAPS markers toward the PH-1 parental type. This suggested that some of the progeny might have been PH-1 macroconidial contaminants. Although this seemed unlikely, given that each single ascospore was collected from the germination plate at 100X magnification, the identities of the eight transgressive strains as bona-fide recombinant progeny was confirmed by examination of additional telomere and repetitive fingerprinting markers (Table 3.3). Analysis of sexual and asexual fecundity demonstrated that the progeny varied significantly in these traits (Figure 3.11, Table 3.4, and Table 3.5).

Pathogenicity tests on Pioneer 2555 were repeated using larger numbers of replications, and the results demonstrated that the four most aggressive strains were not significantly different from one another, but they were all significantly more aggressive than the parents. The four least aggressive strains were all less aggressive than the parents, but these differences were not significant in every case (Figure 3.12A, Table 3.6). Analysis of DON levels in the blighted heads revealed the highly aggressive strains produced

astounding amounts of DON in some cases (Figure 3.12B, Table 3.7). Production of DON by the strains in culture varied, and was not correlated with the results *in planta* (Figure 3.12C, Table 3.8).

**Heritability of aggressiveness:** To determine whether the differences in aggressiveness among the progeny were heritable, three single macroconidial isolates were collected from the most aggressive progeny strain, the least aggressive progeny strain, and the two parental strains. The progenitor strains were included in the experiment as controls. Conidia obtained from these isolates were re-inoculated onto the susceptible Pioneer inbred 2555 (Figure 3.13). Results demonstrated that, with one exception, aggressiveness was heritable (Figure 3.14A and Table 3.9). The exception was one of the single-spored derivatives of the PH-1 strain that had apparently “degenerated” and lost its aggressiveness on Pioneer 2555. None of the single-spored strains had the opposite phenotype, i.e. was significantly more aggressive than its progenitor strain. The results for DON production were much more variable but they did suggest that this trait, too, was heritable to at least some degree (Figure 3.14B and Table 3.10).

**Potential epigenetic sources of variation:** Given the observation that one of the single-spore isolates from PH-1 “degenerated”, and became less aggressive, I decided to investigate the frequency of this occurrence among a larger number of single-spored isolates. Thus, I collected twenty single-spored macroconidial isolates of PH-1 and of Gz3639GFP, and twenty single-spored, ascospore progeny from homothallic matings for comparison. The populations of asexually and sexually derived progenies from both PH-1 and Gz3639GFP were screened on the susceptible Pioneer 2555 variety for aggressiveness. Interestingly, the asexually derived, clonal progeny strains were statistically less aggressive as a group compared with their sexually derived counterparts in both parental backgrounds (Figure 3.15). Additionally, and even more interestingly, the range of symptoms differed dramatically among the two classes. The symptoms induced by the mitotically-derived strains ranged from 0.5 to 5 symptomatic spikelets for PH-1, and from 1 to 5 for Gz3639GFP-derived strains. In contrast, the range for the

meiotically-derived strains was much broader, with some of these progeny producing symptoms that were as severe as nine symptomatic spikelets for PH-1, and 11 symptomatic spikelets for Gz3639GFP-derived strains (Figure 3.15).

### 3.4 Discussion

In chapter 2 of this dissertation, I reported that *G. zeae* isolates causing FHB in Kentucky and Indiana were genetically very diverse. I also reported that it was common to recover more than one genotype from a single blighted wheat head. Similar findings have been reported for other populations of *G. zeae* in North America (Zeller, Bowden, and Leslie, 2003, 2004; Goswami, and Kistler, 2004; Schmale et al., 2006; Gale et al., 2007, 2011). The presence of significant genetic variation is in accordance with the hypothesis that out-crossing occurs frequently in *G. zeae* field populations (Gale et al., 2007; Gale et al., 2002; Miedaner et al., 2001; Schmale et al., 2006; Zeller et al., 2003). It is known that *G. zeae* has the ability to out-cross in the laboratory (Bowden and Leslie, 1999). Significant variation in aggressiveness among field populations of *G. zeae* has been reported in several previous studies (Akisanmi, Backhouse, Simpfendorfer, and Chakraborty, 2006; Goswami and Kistler, 2004; Miedaner, Cumagun, and Chakraborty, 2008). In this chapter of my dissertation, my goal was to address the hypothesis that crosses among different *G. zeae* genotypes could yield progeny with significantly higher levels of pathogenicity or fitness than either parent (transgressive strains).

As a result of its economic importance, significant resources have been devoted to understanding the biology, genetics, and population biology of *G. zeae*, including a recently completed project to sequence the genomes of two lineage 7 strains (Cuomo, 2007; Cuomo et al., 2005). The two strains chosen for genome sequencing, PH-1 and Gz3639, are the two most commonly used lab strains for basic biological research on pathogen biology, genetics, and mycotoxin production. There is a lot of “conventional wisdom” regarding these strains, but they have never, to my knowledge, been directly compared in a published study. I decided to make my cross between Gz3639 and PH-1 because of the availability of extensive genomic and genetic resources for both. This

opens up the possibility for future identification of individual segregating genes with effects on pathogenicity, if they exist. As an additional benefit, a list of approximately 10,000 SNPs that differentiate the two strains has been made available (Cuomo et al., 2007). These can be used in the future to produce a saturated genetic map and to study linkage relationships. For my dissertation, I utilized the SNP list to design CAPs markers that I used to confirm the recombinant identity of the progeny.

I began my work by comparing the two parent strains, PH-1 and Gz3639. The genomes of the two overall are reported to be very similar (Cuomo et al., 2007). Nonetheless, they differed in many genetic markers (Chapter 2 of this Dissertation), and also in some important pathogenicity-related traits, most notably in ascospore production and trichothecene production, both of which have been related to production of FHB on susceptible wheat varieties in the field (Burlakoti et al., 2007; Maier et al., 2006; Ohe et al., 2010; Paul, Lipps, and Madden, 2005; Stein et al., 2009). There were no significant differences between the two strains in their aggressiveness on SRWW in the greenhouse.

Because *G. zeae* is homothallic (Bowden and Leslie, 1999), most perithecia resulting from a confrontation between two strains actually come from "selfing", and only a few are the result of out-crossing. To facilitate identification of out-crossed perithecia, I utilized a fluorescent labeling approach. One of the parents, Gz3639, was available as a tagged version expressing a gene encoding green fluorescent protein. Tiana Madry, an undergraduate in the lab, produced this strain, which I named Gz3639GFP. All of the fluorescent cirrhi I collected on the PH-1 (non-fluorescent) side of the crossing plates turned out to be the result of out-crossing. To exclude the possibility that GFP-tagging influenced the traits under study, I included both Gz3639 and Gz3639GFP as controls in all my experiments. The GFP-tagged transformant behaved similarly to the WT progenitor strain in all cases.

FHB symptoms produced by the progeny on a susceptible SRWW variety Pioneer 2555 reflected a normal distribution of pathogenicity phenotypes, skewed toward the low end, and ranging from almost non-pathogenic (producing symptoms similar to those seen in the presence of type-II resistance), to highly aggressive. Among the 95 progeny tested, four strains (4.2%) were statistically significantly more aggressive than either of the

parental strains, and were thus classified as transgressive segregants. Recently an article was published that reported results that were consistent with my results here. Voss et al. (2010) tested 120 progeny derived from two separate crosses between highly aggressive parental strains on winter wheat under field conditions across four locations. They identified 1.2% of the progeny as transgressive segregants that were significantly more aggressive than the parents.

The continuous variation I observed could be the result of several genes segregating, but it could also result from environmental variation. However, the phenotypes of the transgressive segregating isolates were heritable in mitotic (macroconidial) progeny, and this supports the idea that the cause of the variation is genetic, and not environmental.

One of two identified QTL loci related to aggressiveness in *G. zeae* has been mapped to the trichothecene biosynthetic cluster that is responsible for production of DON (Cumagun et al., 2004). The PH-1 and Gz3739 strains are both 15-ADON strains, and they contain the same trichothecene cluster (Alexander et al., 2011). Nevertheless, they varied significantly in their ability to produce DON both *in planta* and in culture, possibly as a result of variation in modifier genes outside of the cluster. There was a relationship among their progeny between aggressiveness and DON production: highly aggressive strains produced very high amounts of DON *in planta*, while the non-aggressive strains produced very little. This relationship between DON and FHB symptom severity has been reported previously (Burlakoti et al., 2007; Otto et al., 2002; Paul et al., 2005). Published data suggest that DON is not an essential pathogenicity factor, but does play an important role in inhibition of host resistance reactions so that the fungus can efficiently colonize the infected spike (Maier et al., 2006). Proctor et al. (1995) showed that a non-trichothecene producing mutant strain was still pathogenic, but it was significantly reduced in aggressiveness. While there was an association between DON production *in planta* between aggressiveness and DON production, the same was not true for DON production in liquid medium. Thus, the causes of variation in DON production *in planta* may be related to genes other than those involved directly in biosynthesis, e.g. genes involved in responses to plant signals. The two parent strains, in spite of producing different amounts of DON, did not differ in their aggressiveness to



SRRW. Thus, factors other than DON are also likely to be important in determining aggressiveness among the progeny.

In addition to the FHB aggressiveness phenotype I observed a high degree of variability in both sexual and asexual fecundity. These traits did not appear to be associated with aggressiveness in the greenhouse assay. In the field, an increase in fecundity could increase the fitness of that genotype, and in the absence of any negative selective pressure, to result in a relative increase of that genotype in the population.

I cannot conclude that the transgressive segregating strains generated in my study would persist, or ultimately cause increased levels of FHB, under field conditions. Voss et al. (2010) reported that the highly aggressive transgressive strains produced in their study persisted in the population only during seasons when conditions for disease development were unfavorable, and they did not persist with a high frequency during years with favorable conditions for FHB.

The factors that are important for changes in aggressiveness among the progeny are heritable in asexual progeny, and thus they may be linked to genes that have recombined during the cross. However, it is important to note that selfed ascospore progeny, which presumably derive from the combination of two identical (self) genomes, also displayed a broad range of aggressiveness and fecundity phenotypes, although selfing did not give rise to statistically different transgressive progeny. We would not expect recombination of genes to play a role in the progeny recovered from selfed perithecia. However, this is assuming that all nuclei are identical in the selfing individual. In fact, due to mutation, not all nuclei will be genetically identical. Perhaps the processes associated with sexual cycle result in an increase of mutation rate in some genotypes, which could cause genome instability and variability in "selfed" progeny. Another possibility is that epigenetic factors [e.g. DNA methylation (appendix 2) or chromatin remodeling] that occur during meiosis may play a role in generating variation. This very interesting possibility requires further investigation, since it would imply that every meiosis, whether selfed or out-crossed, has the potential to produce phenotypes that could be acted upon by selective forces in the field.

### 3.5 References

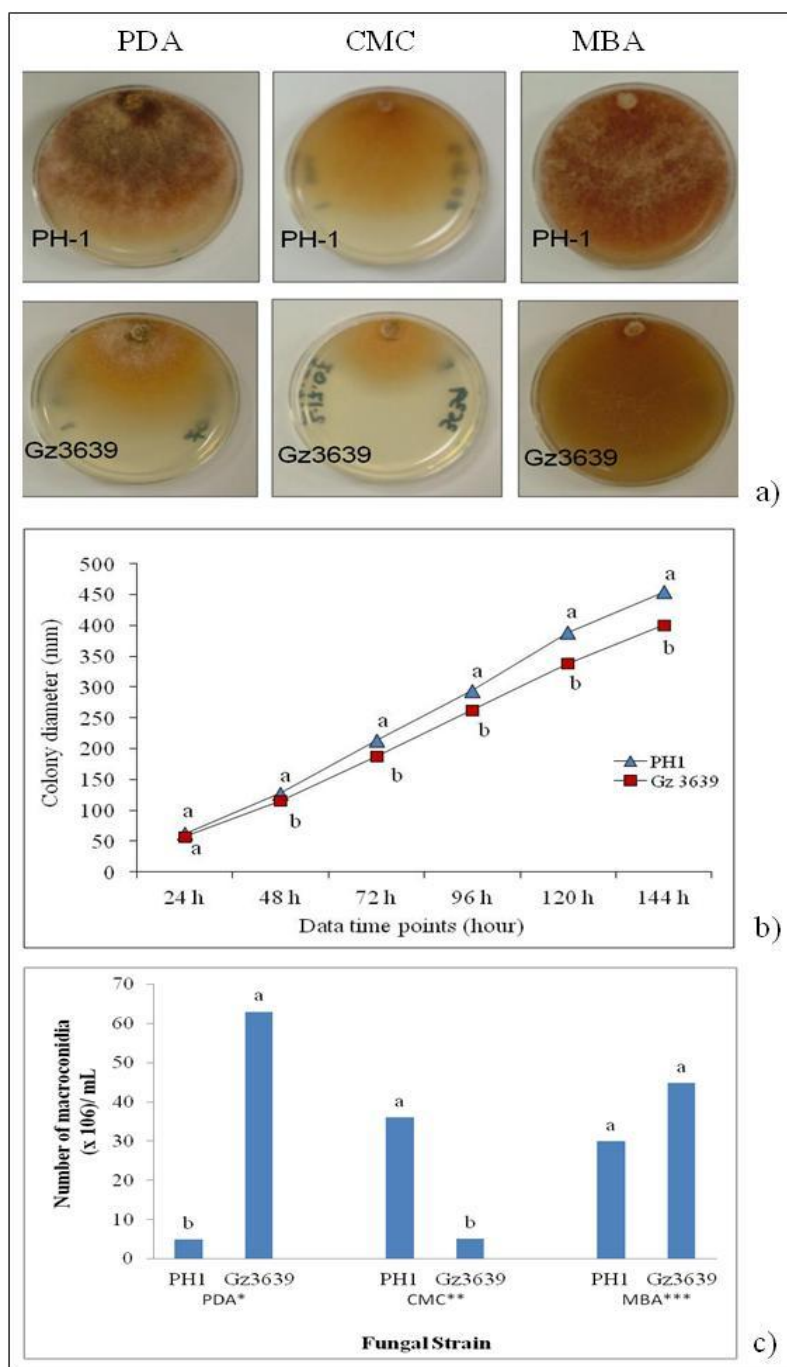
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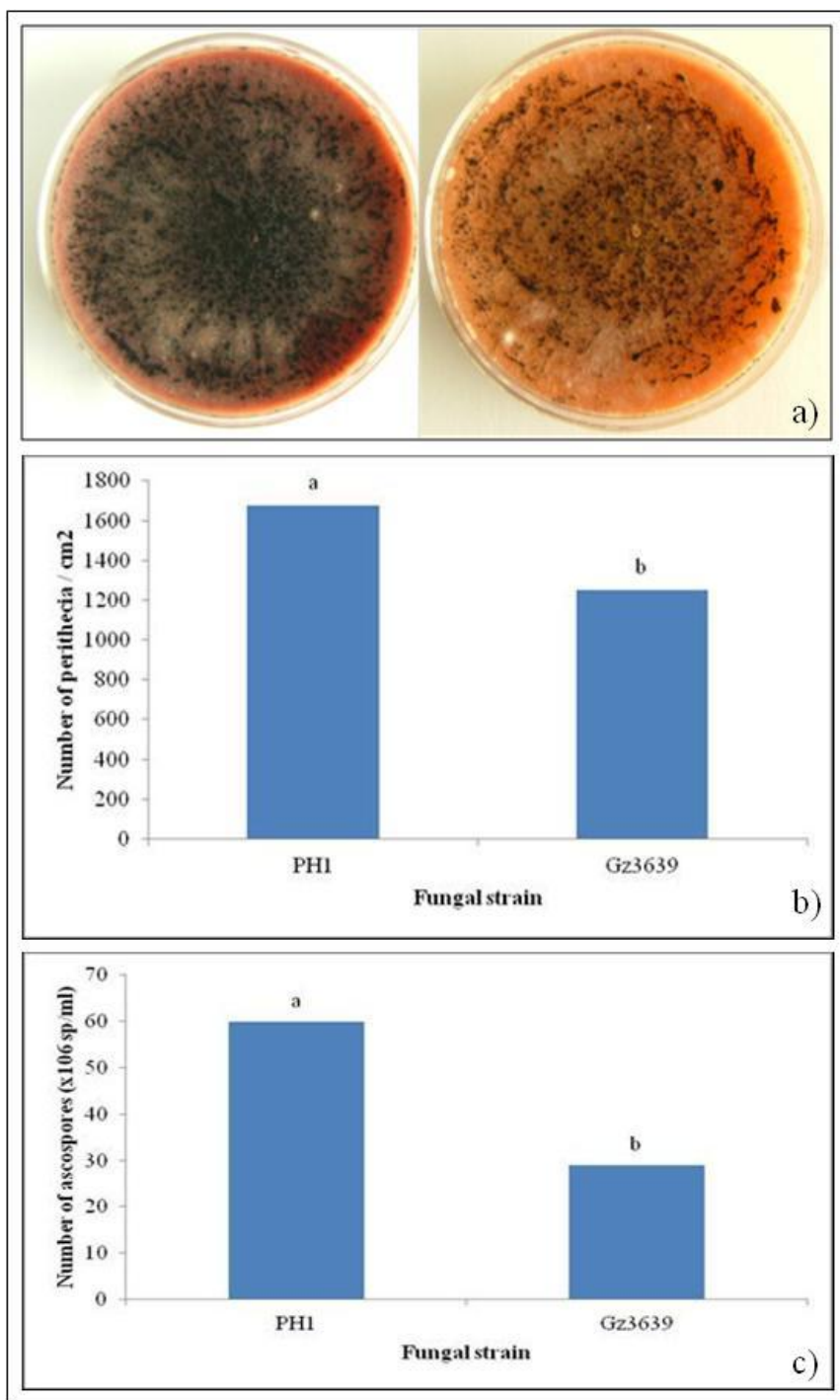
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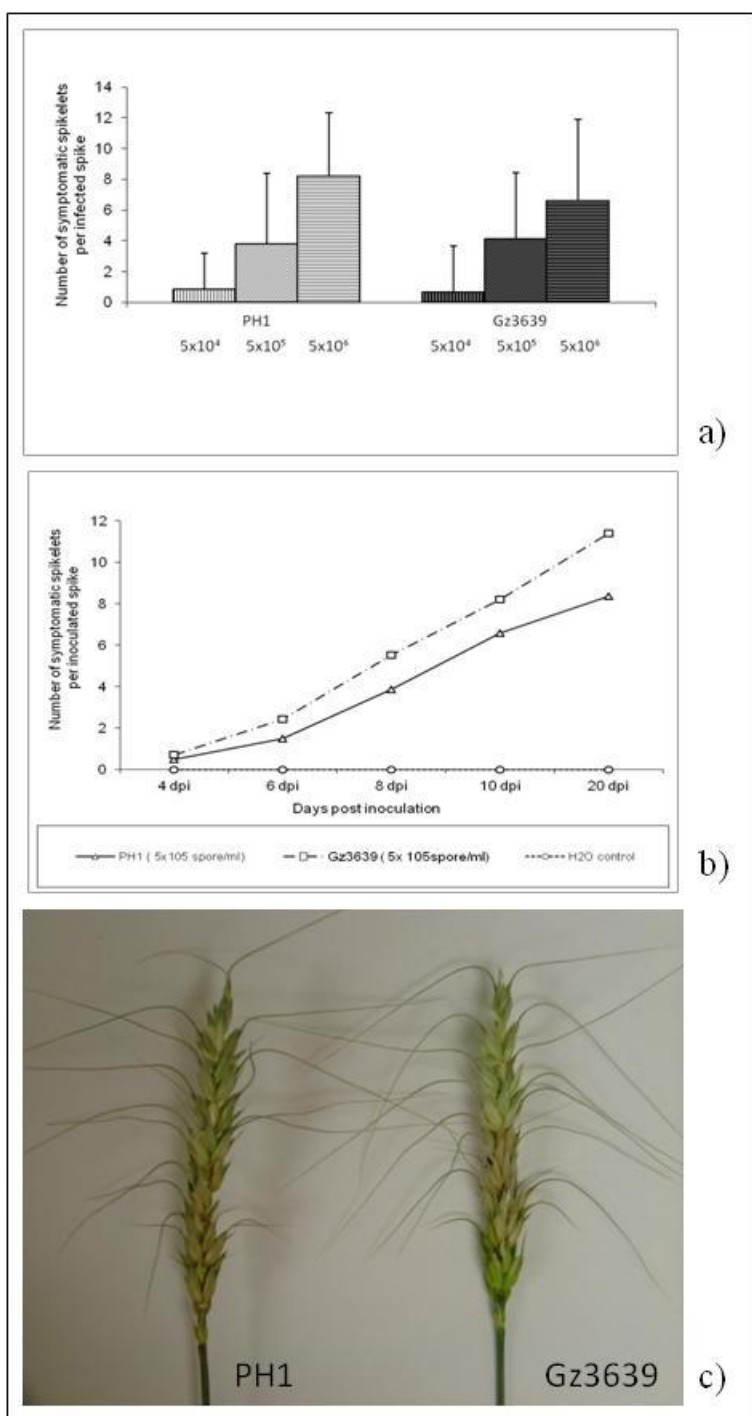


**Figure 3.1.** Comparison of *G. zeae* strains PH-1 and Gz3639 in culture. a) colony morphology on different media; b) radial growth averaged over the three media, and asexual (macroconidial) spore production c).

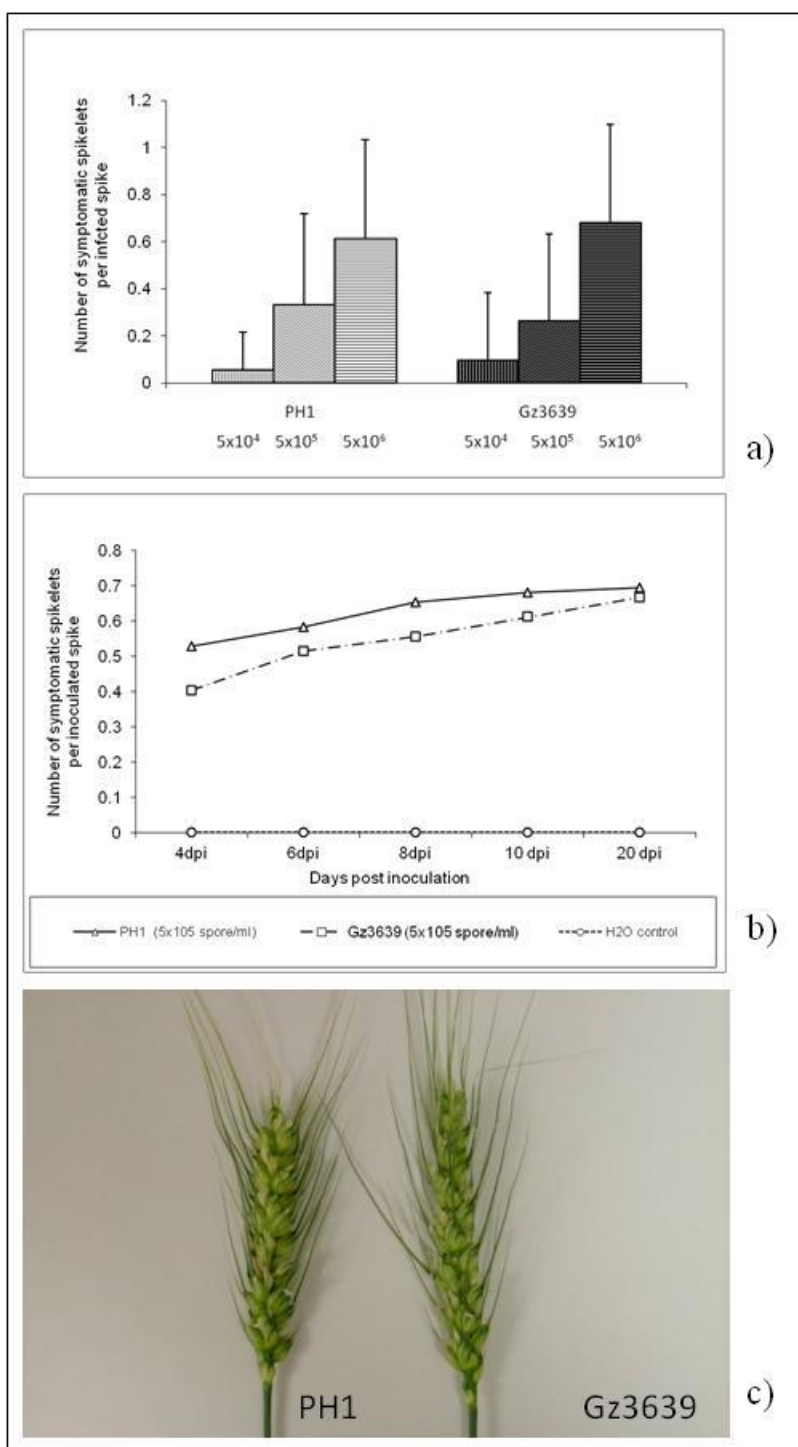


**Figure 3.2.** Comparison of the fertility of strains PH-1 and Gz3639. a) Sexual development on carrot agar; b) relative numbers of perithecia; and c) relative numbers of ascospores produced after 21 days growth on carrot agar media.

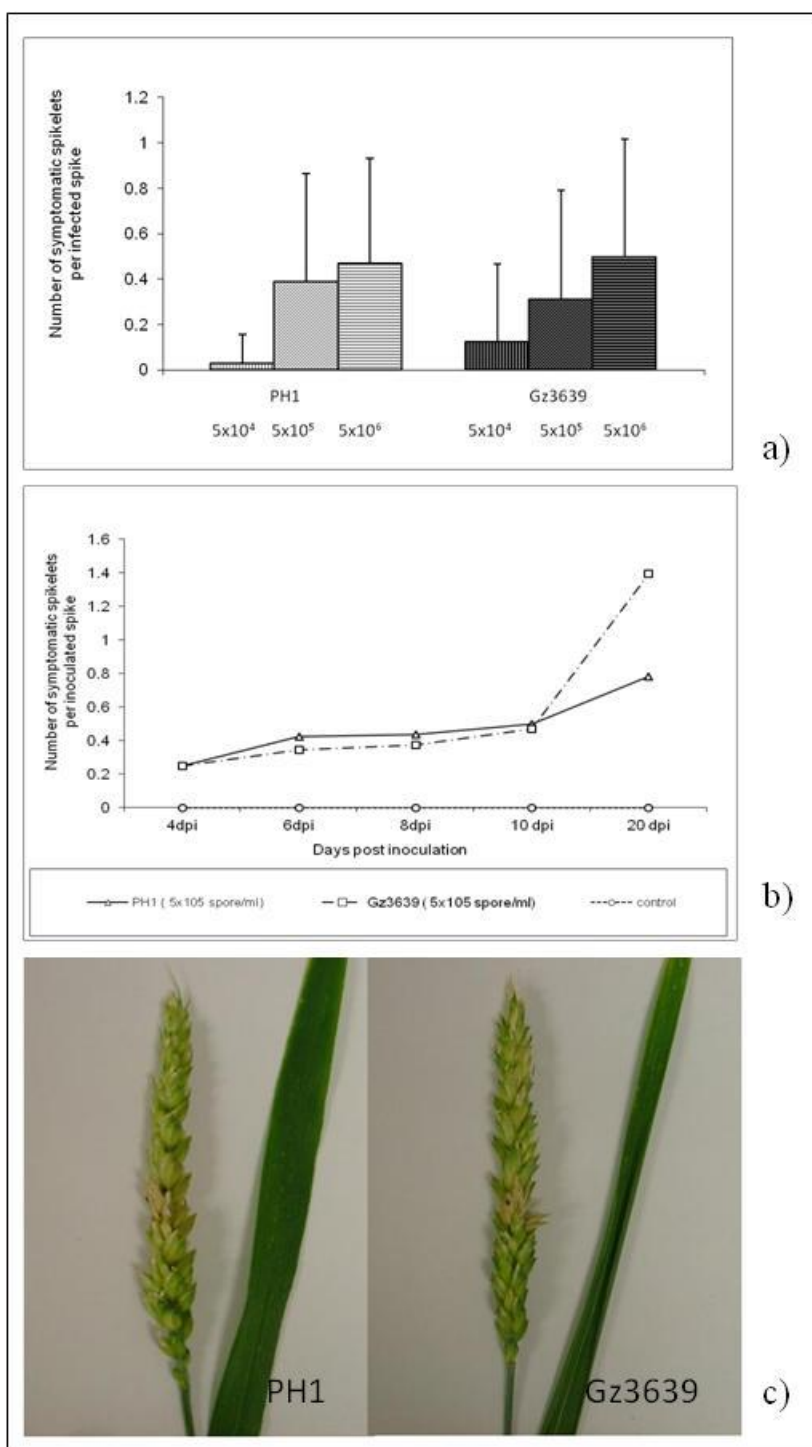




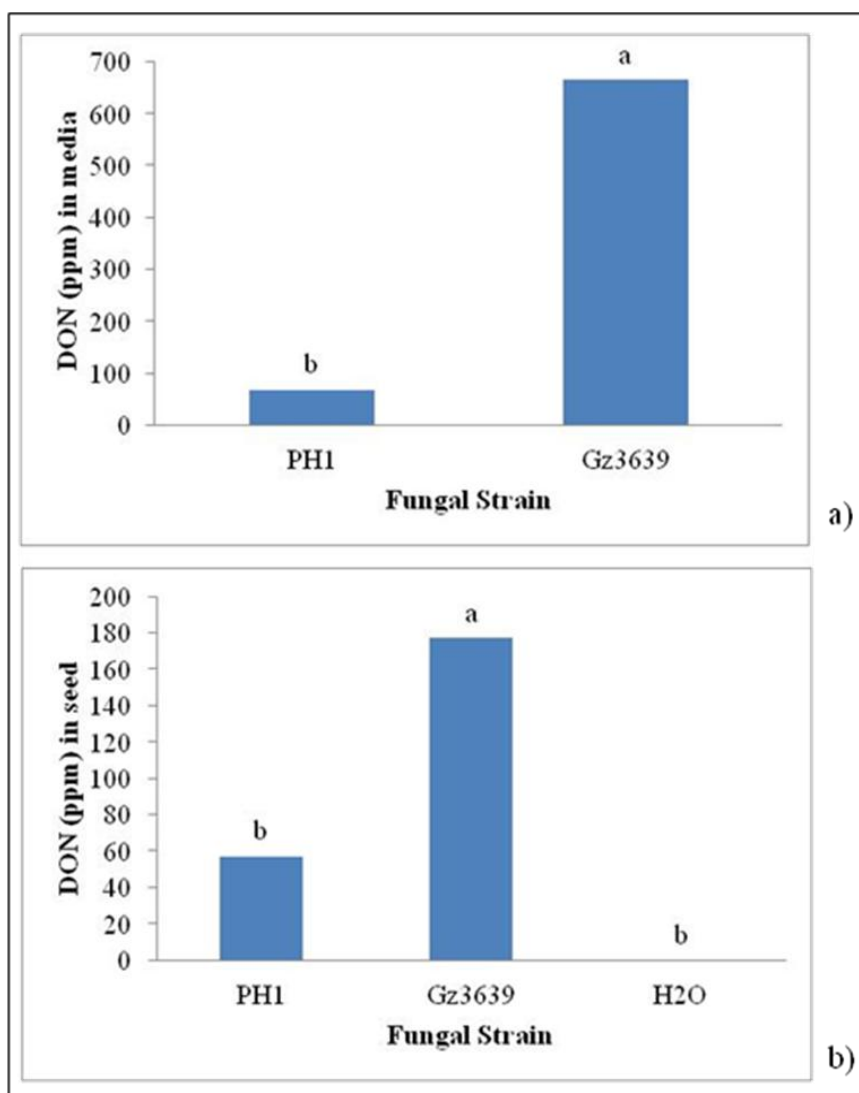
**Figure 3.3A:** Comparison of PH-1 and Gz3639 aggressiveness level on susceptible wheat variety Pioneer 2555. a) Effect of spore concentration on 7 days post infection (dpi) FHB symptom development; b) FHB symptom development and disease progress curve over the period of 20 dpi; and c) 10 dpi external FHB symptoms.



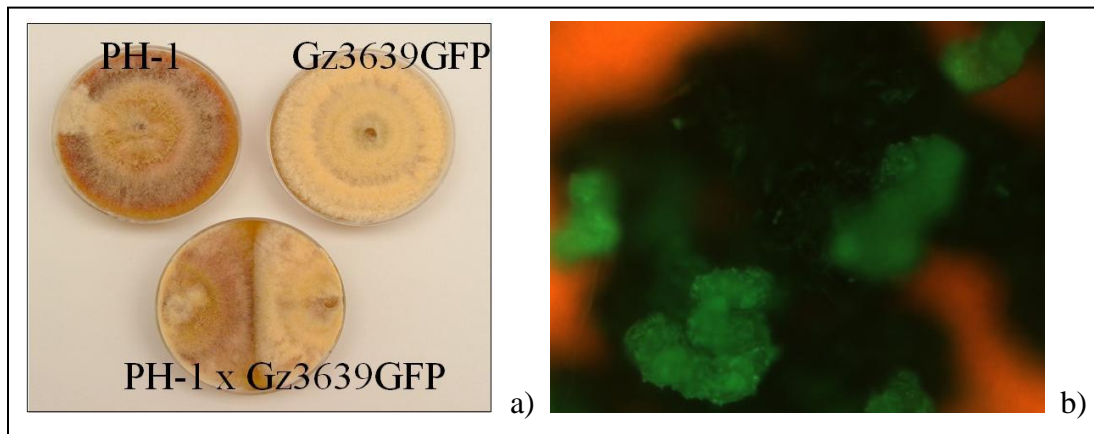
**Figure 3.3B:** Comparison of PH-1 and Gz3639 aggressiveness level on wheat variety with Type II resistance Pioneer 25R18. a) Effect of spore concentration on 7 days post infection (dpi) FHB symptom development; b) FHB symptom development and disease progress curve over the period of 20 dpi; and c) 10 dpi external FHB symptoms.



**Figure 3.3C:** Comparison of PH-1 and Gz3639 aggressiveness level on the moderately resistant wheat variety Truman. a) Effect of spore concentration on 7 days post infection (dpi) FHB symptom development; b) FHB symptom development and disease progress curve over the period of 20 dpi; and c) 10 dpi external FHB symptoms.



**Figure 3.4.** Comparison of mycotoxin production by *G. zeae* strains PH-1 and Gz3639 in: a) liquid medium (2% GYEPD); and b) *in planta*.



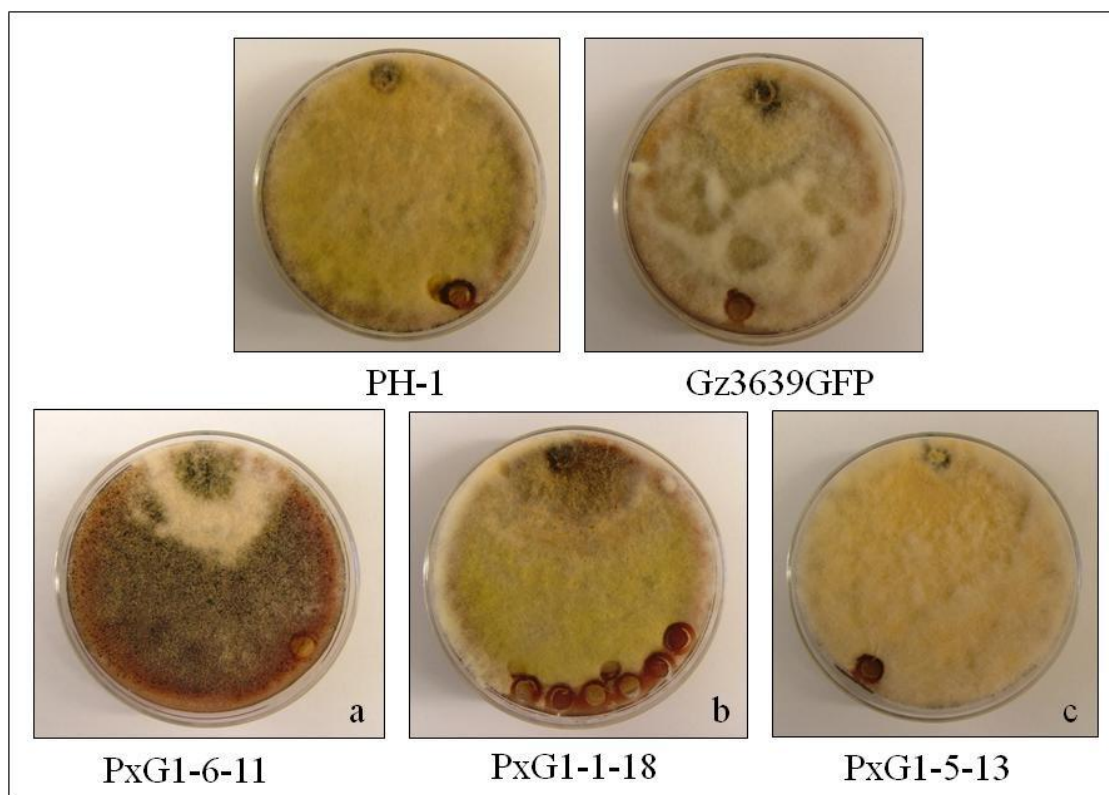
**Figure 3.5:** The mycelial plug method was used to cross PH-1 and Gz3639GFP. a); Putative heterothallic perithecia were initially selected based on visualization of cirrhi containing spores expressing GFP on the PH-1 side of the crossing plate b).

**Table 3.1:** List of PCR primers used to create molecular probes for the study of genotypic and phenotypic diversity created through the sexual cross PH-1xGz3639GFP.

Primer	Primer Sequence	Marker type	Reference
PstI 1F	5' TATGAAGAGCTGGACTGCATGGC 3'	CAP	this study
PstI 1R	5' TAACCCAGGGAGCGACTATTTGC 3'	CAP	this study
PstI 2F	5' TCGACTTGAGAGACAACCGAGCA 3'	CAP	this study
PstI 2R	5' TCAAAGGGCTTGGAGGAGAGTCA 3'	CAP	this study
EcoRI 3F	5' GGTTTCGGTGAGTCTTTAAGCCCC 3'	CAP	this study
EcoRI 3R	5' CGGCTTGAGGGTTTTTCGAGC 3'	CAP	this study
EcoRI 4F	5' TATCGCAGCAAAATGTGCAGGG 3'	CAP	this study
EcoRI 4R	5' GCCCCTCATTTGATCAAGCCTT 3'	CAP	this study
REP1.52F	5' GGTTTCATGAAGACATTGGTC 3'	RFLP	this study
REP1.52R	5' TGATCCCGGTCTACTACATC 3'	RFLP	this study
REP1.92F	5' GTCACCAAAGTTCGGTTCT 3'	RFLP	this study
REP1.92R	5' CGGTTATGCAATCTGTTCTT 3'	RFLP	this study
TELO F	5' TTAGGGTTAGGGTTAGGG 3'	RFLP	Schechtman, 1990
TELO R	5' CCCTAACCCTAACCCTAA 3'	RFLP	Schechtman, 1990

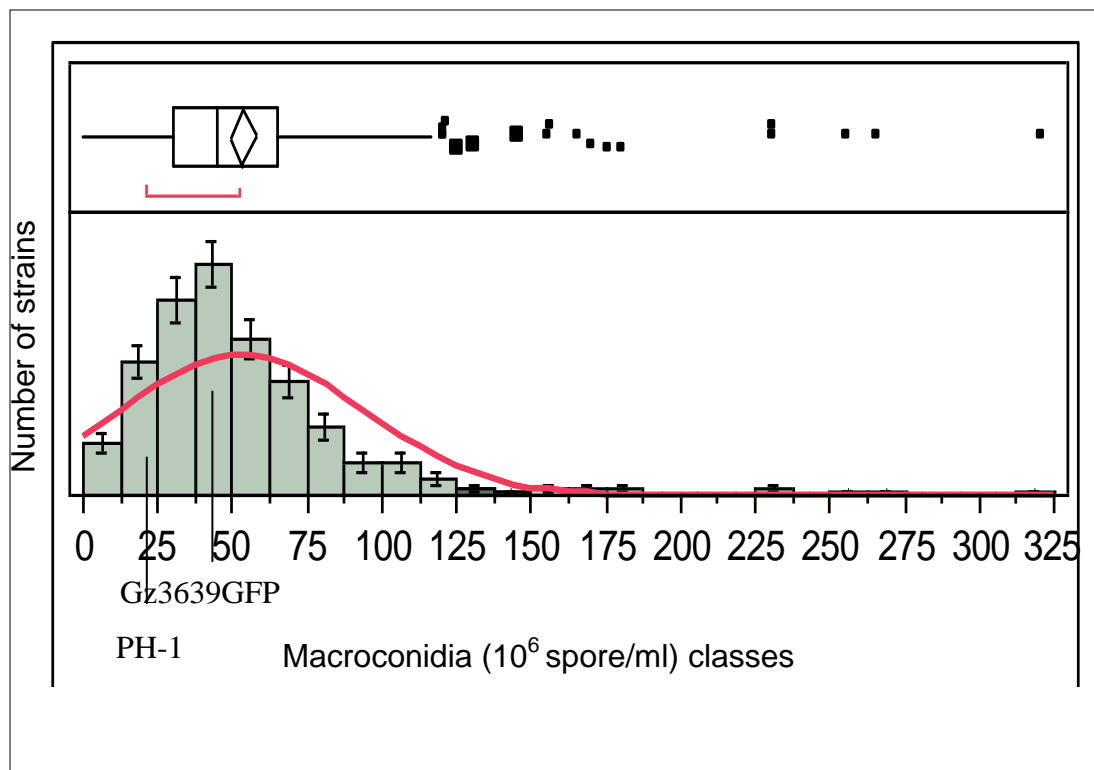
**Table 3.2:** Segregation analysis of EcoRI3 and PstI2 CAPs markers used to determine genetic identity of four randomly selected putative heterothallic perithecia.

			1:1 segregation			
	Perithecium	PH1:Gz3639	Total	Df	$\chi^2$	P
EcoRI3	Per#1	14:10	24	23	0.667	0.999
	Per#2	17:6	23	22	5.083	0.999
	Per#5	10:14	24	23	0.667	0.999
	Per#6	14:10	24	23	0.667	0.999
PstI2	Per#1	16:8	24	23	2.667	0.999
	Per#2	18:5	23	22	7.083	0.998
	Per#5	10:14	24	23	0.667	0.999
	Per#6	15:9	24	23	1.500	0.999
			1:1:1:1 segregation			
	Perithecium	PH1:Gz3639:PH1:Gz36339	Total	Df	$\chi^2$	P
EcoRI3 + PstI2	Per#1	14:10:16:8	48	47	3.333	0.999
	Per#2	17:6:10:14	27	26	12.167	0.999
	Per#5	10:14:10:14	28	27	1.333	0.999
	Per#6	14:10:15:9	48	47	2.167	0.999

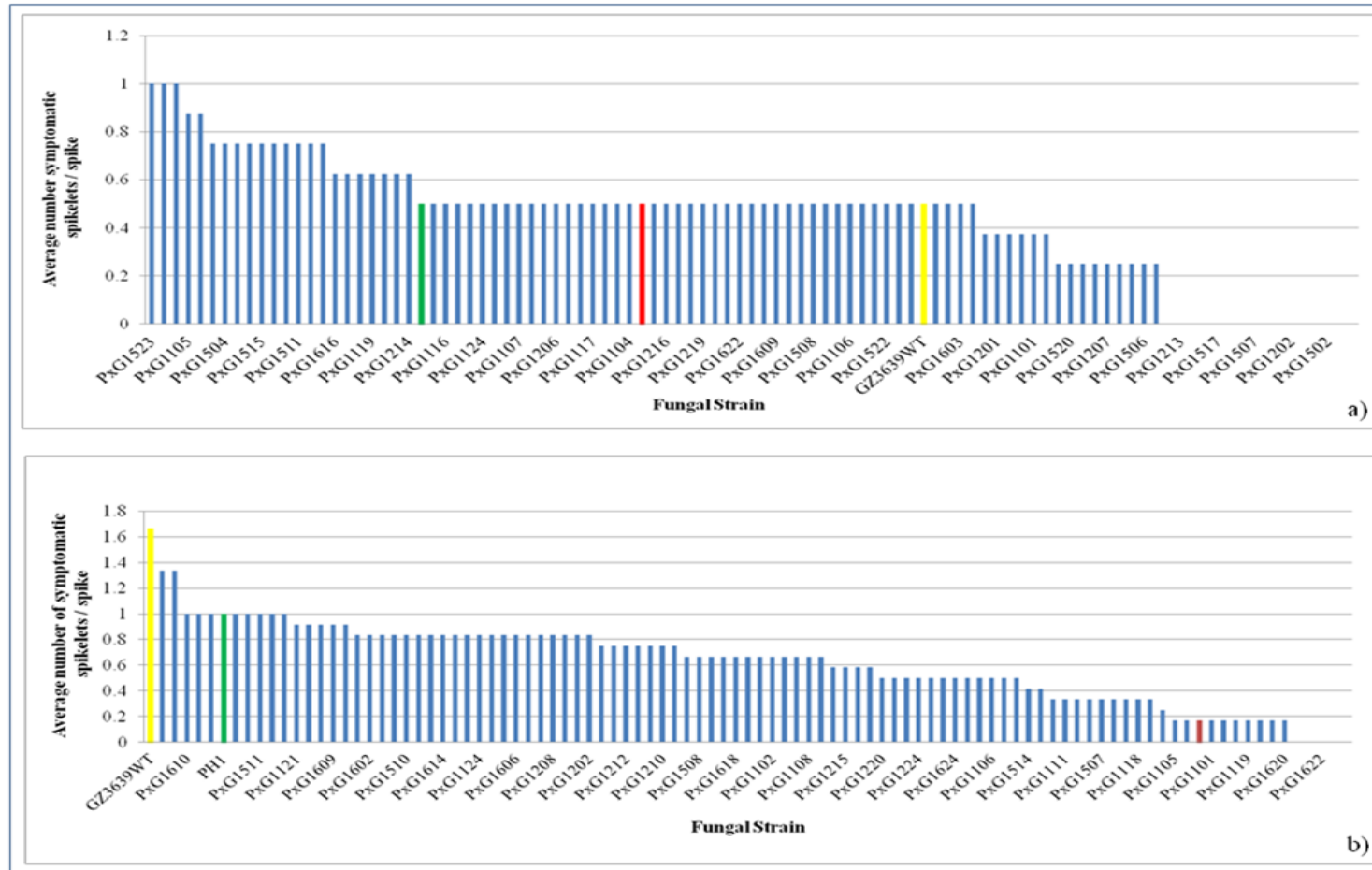


**Figure 3.6.** Examples of morphological variability among PxG progeny strains grown on PDA. a) Abundant production of perithecia on this non-inducing medium; b) production of pigment (aurofusarin and rubrofusarin); c) thick aerial mycelium producing very few macroconidia.

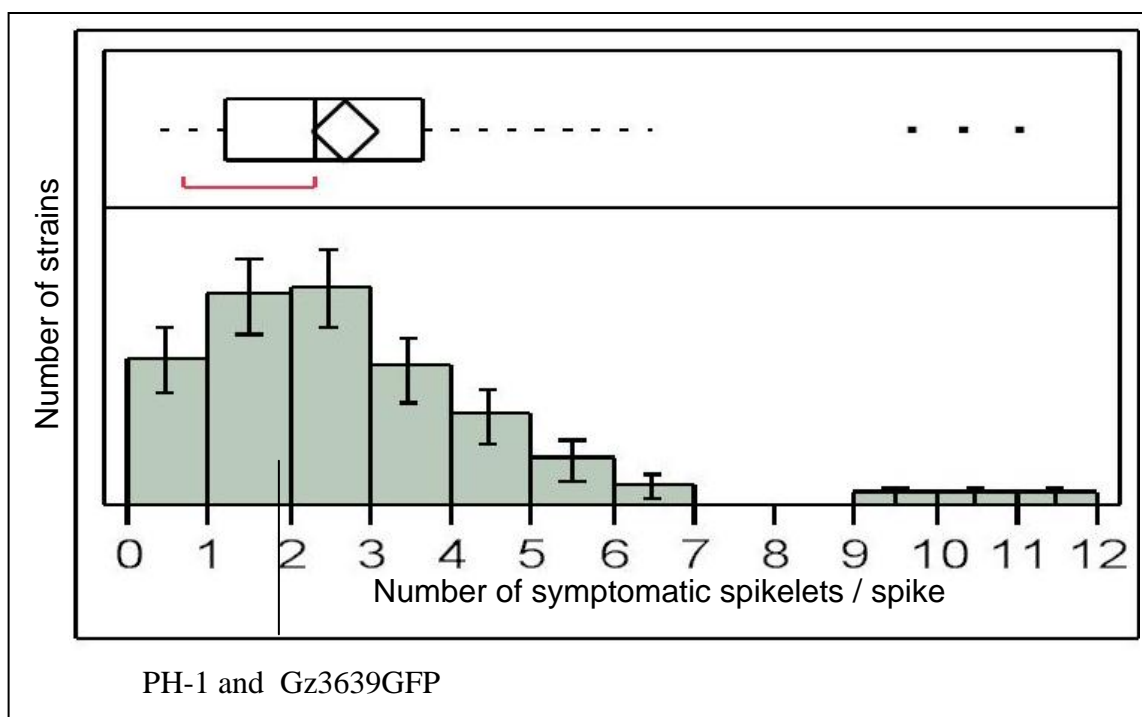




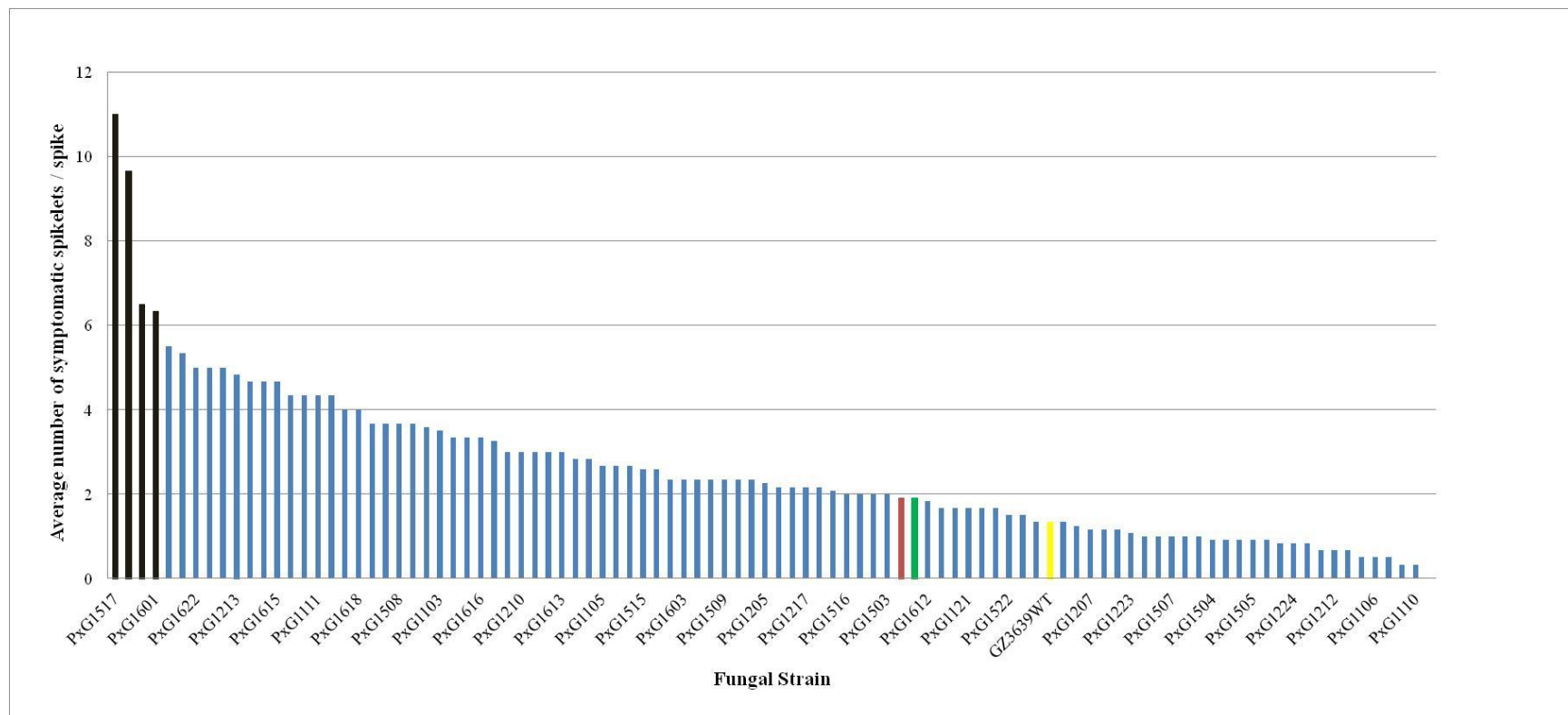
**Figure 3.7.** Frequency distribution of the macroconidial production among 95 progeny strains from the cross between PH-1 and Gz3639GFP.



**Figure 3.8:** FHB symptoms 7dpi of 95 PxG progeny strains and the parental strains on: a) Pioneer 25R18 (a variety with Type II resistance), and b) Truman (a moderately resistant variety). The parental strains PH-1 and Gz3639GFP are indicated with a green and a red bar, respectively, and WT Gz3639 is indicated with a yellow bar. Each bar represents the average of three observations. There were no statistical differences among the strains in these experiments.



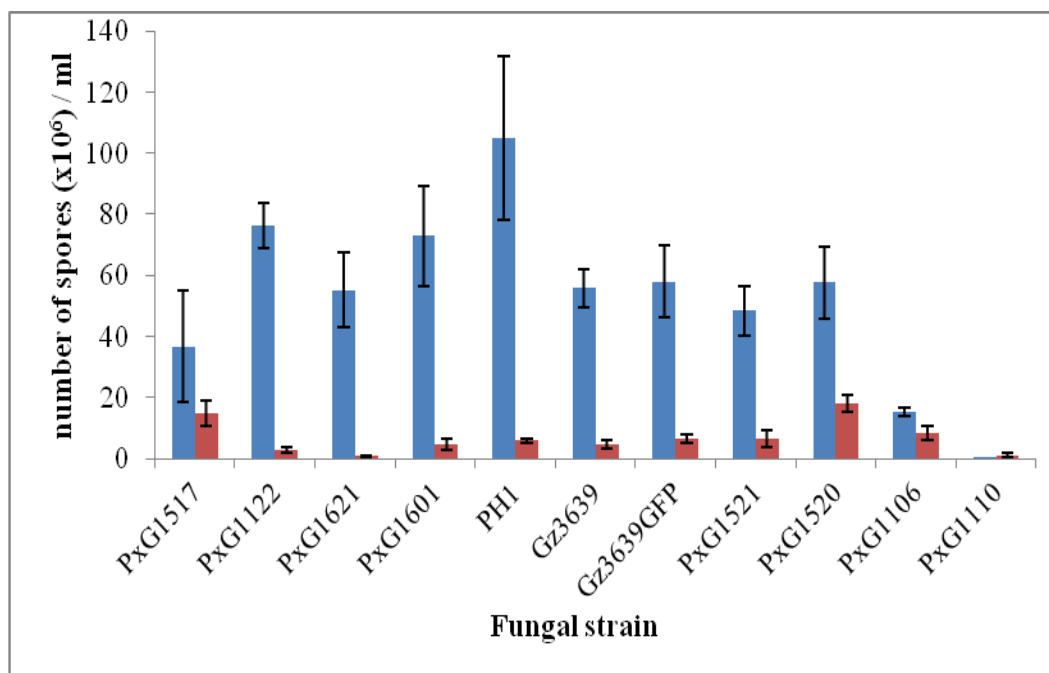
**Figure 3.9.** Frequency distribution of FHB aggressiveness levels 7dpi among progeny strains from the cross between PH-1 and Gz3639 on the susceptible variety Pioneer 2555.



**Figure 3.10:** The initial screen of PXG progeny based on 7dpi FHB symptom severity on the susceptible winter wheat variety Pioneer 2555. The parental strains PH-1 and Gz3639GFP are indicated with a green and a red bar, respectively, and the Gz3639 WT strain is indicated with a yellow bar. The fungal strains significantly different from both parental strains are denoted by black bars. Each bar presents the average of three observations.

**Table 3.3:** Recombination of molecular markers confirming the recombinant nature of the strains generated through PH-1 x Gz3639GFP cross.

Strain	Allele	PH1	Gz3639GFP	Strain	Allele	PH1	Gz3639GFP
PxG1517	REP192BA3k		+	PxG1110	REP192BA3k	+	
	REP192BA0.8k	+			REP192BA0.8k		+
	TELOSM650		+		TELOSM650	+	
	TELOSM800	+			TELOSM800	+	
	TELOSM4500		+		TELOSM4500		+
	TELOSM200	+			TELOSM200	+	
	TELOBA1000	+			TELOBA1000	+	
	TELOBA500		+		TELOBA500	+	
	EcoRI3		+		EcoRI3	+	
	PstI2		+		PstI2	+	
PxG1122	REP192BA3k	+		PxG1520	REP192BA3k	+	
	REP192BA0.8k		+		REP192BA0.8k		+
	TELOSM650	+			TELOSM650	+	
	TELOSM800		+		TELOSM800	+	
	TELOSM4500	+			TELOSM4500	+	
	TELOSM200		+		TELOSM200		+
	TELOBA1000	+			TELOBA1000	+	
	TELOBA500	+			TELOBA500	+	
	EcoRI3	.			EcoRI3	+	
	PstI2	.			PstI2	+	
PxG1621	REP192BA3k	+		PxG1521	REP192BA3k		+
	REP192BA0.8k		+		REP192BA0.8k	+	
	TELOSM650	+			TELOSM650		+
	TELOSM800	+			TELOSM800		+
	TELOSM4500		+		TELOSM4500		+
	TELOSM200	+			TELOSM200		+
	TELOBA1000	+			TELOBA1000		+
	TELOBA500	+			TELOBA500		+
	EcoRI3		+		EcoRI3	+	
	PstI2	+			PstI2		+
PxG1601	REP192BA3k		+	PxG1106	REP192BA3k		+
	REP192BA0.8k	+			REP192BA0.8k	+	
	TELOSM650		+		TELOSM650		+
	TELOSM800	+			TELOSM800	+	
	TELOSM4500		+		TELOSM4500		+
	TELOSM200		+		TELOSM200	+	
	TELOBA1000		+		TELOBA1000		+
	TELOBA500		+		TELOBA500		+
	EcoRI3	+			EcoRI3		+
	PstI2		+		PstI2		+



**Figure 3.11:** Comparison of ascospore (blue bars) and macroconidia (red bars) production on carrot agar between transgressive progeny and parental strains.

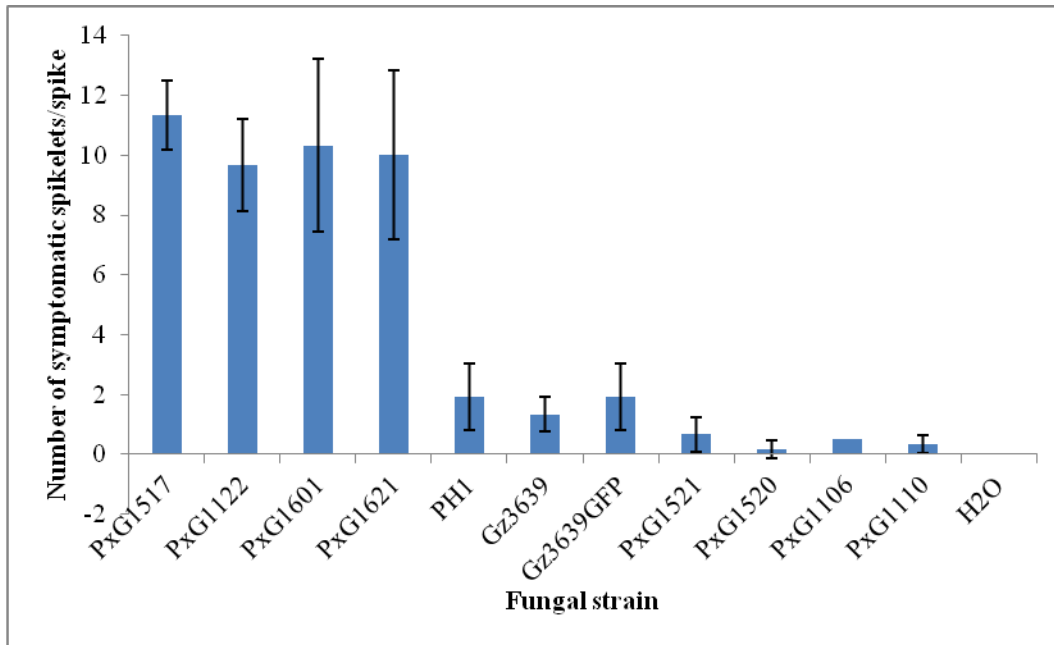
**Table 3.4:** Table: LSD matrix of the pair-wise comparisons for the effect of fungal strain on sexual fecundity. The values in red indicate a statistical difference at the level of  $P \leq 0.05$ .

i/j	PH-1	PxG1521	PxG1106	Gz3639	Gz3639GFP	PxG1601	PxG1122	PxG1621	PxG1601	PxG1110	PxG1520
PH-1		<b>0.0026</b>	<b>&lt;.0001</b>	<b>0.008</b>	<b>0.0111</b>	<b>0.0004</b>	0.1124	<b>0.0073</b>	0.0766	<b>&lt;.0001</b>	<b>0.0106</b>
PxG1521			0.0668	0.6762	0.5865	0.5149	0.1208	0.7004	0.172	<b>0.0089</b>	0.5994
PxG1106				<b>0.0263</b>	<b>0.0194</b>	0.2277	<b>0.0012</b>	<b>0.0284</b>	<b>0.0021</b>	0.3946	<b>0.0203</b>
Gz3639					0.8991	0.2874	0.2516	0.9738	0.3382	<b>0.0029</b>	0.914
Gz3639GFP						0.2349	0.3066	0.8731	0.405	<b>0.002</b>	0.985
PxG1517							<b>0.0304</b>	0.3023	<b>0.0469</b>	<b>0.0432</b>	0.2422
PxG1122								0.2385	0.8473	<b>&lt;.0001</b>	0.298
PxG1621									0.3221	<b>0.0032</b>	0.888
PxG1601										<b>0.0002</b>	0.3946
PxG1110											<b>0.0021</b>
PxG1520											

**Table 3.5:** LSD matrix of the pair-wise comparisons for the effect of fungal strain on asexual fecundity. The values in red indicate statistical difference at the level of  $P \leq 0.05$ .

i/j	PH-1	PxG1521	PxG1106	Gz3639	Gz3639GFP	PxG1601	PxG1122	PxG1621	PxG1601	PxG1110	PxG1520
PH-1		0.8471	0.4105	0.7061	0.8256	<b>0.0046</b>	0.3034	0.0999	0.7143	0.1282	<b>0.0003</b>
PxG1521			0.5274	0.5693	0.978	<b>0.0077</b>	0.2233	0.0675	0.5767	0.0881	<b>0.0005</b>
PxG1106				0.2326	0.5454	<b>0.0363</b>	0.0679	<b>0.0158</b>	0.2368	<b>0.0217</b>	<b>0.0029</b>
Gz3639					0.5509	<b>0.0016</b>	0.5114	0.1998	0.9912	0.2478	<b>&lt;.0001</b>
Gz3639GFP						<b>0.0082</b>	0.2133	0.0637	0.5582	0.0834	<b>0.0005</b>
PxG1517							<b>0.0002</b>	<b>&lt;.0001</b>	<b>0.0016</b>	<b>&lt;.0001</b>	0.3242
PxG1122								0.5256	0.5044	0.6131	<b>&lt;.0001</b>
PxG1621									0.1961	0.8967	<b>&lt;.0001</b>
PxG1601										0.2435	<b>&lt;.0001</b>
PxG1110											<b>&lt;.0001</b>
PxG1520											

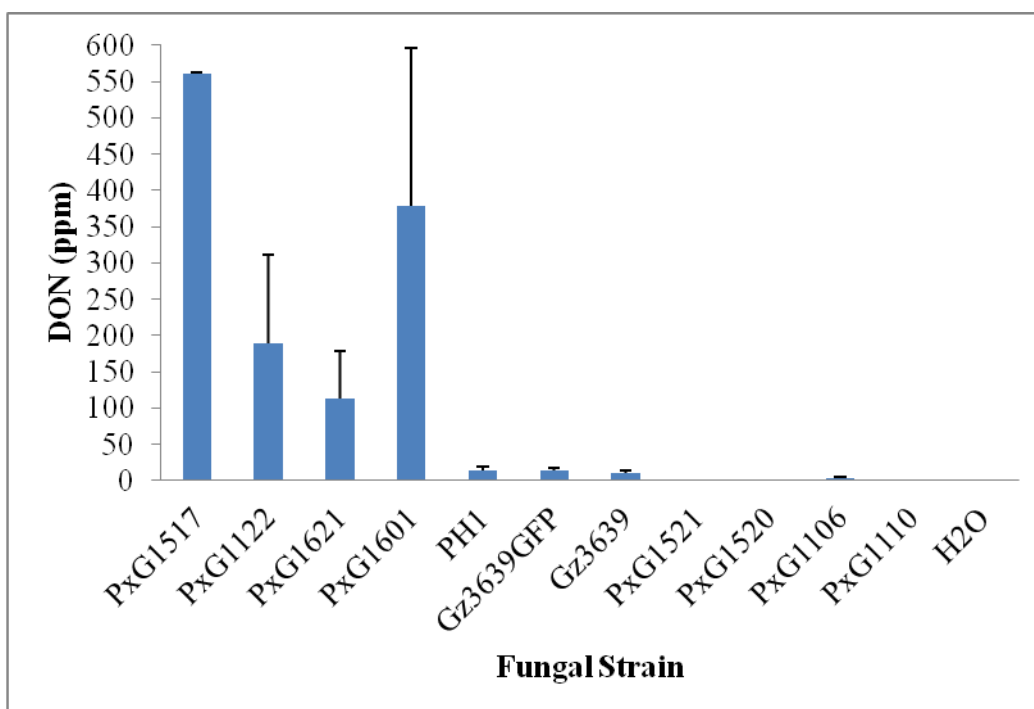




**Figure 3.12A:** Comparison of average aggressiveness among parental strains PH-1 and Gz3639GFP and transgressive progeny strains. Aggressiveness is expressed as the average number of FHB symptomatic spikelets per spike at 7 dpi.

**Table 3.6:** LSD matrix of the pair-wise comparisons for the effect of fungal strain on FHB aggressiveness (expressed as 7 dpi FHB symptom severity). The values in red indicate statistical difference at the level of  $P \leq 0.05$ .

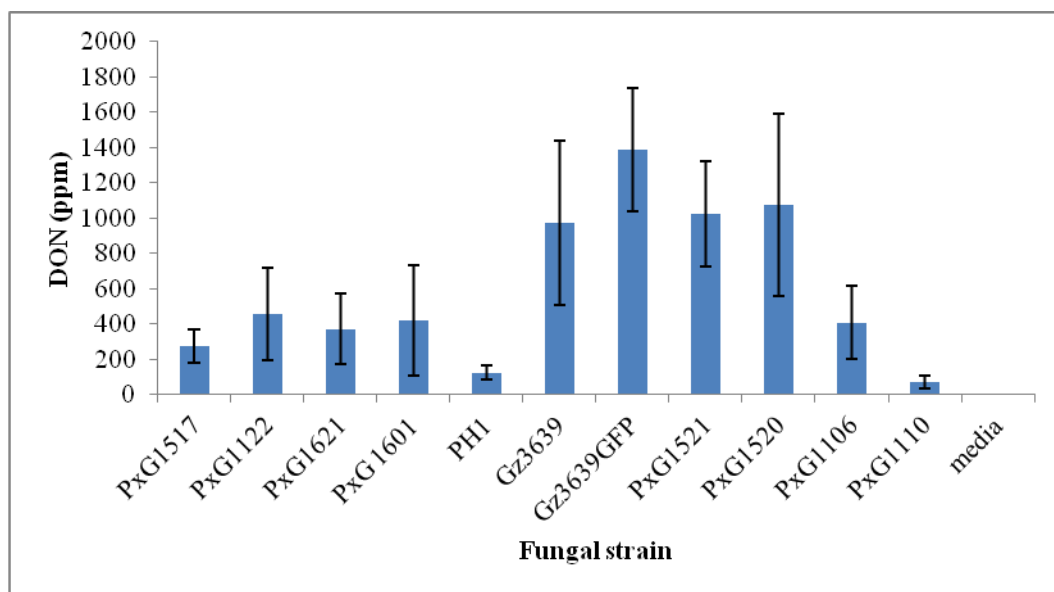
i/j	H2O	PxG1520	PxG1106	PxG1110	PH-1	Gz3639GFP	Gz3639	PxG1517	PxG1122	PxG1601	PxG1621	PxG1521
H2O		0.8153	0.7263	0.8153	0.1872	0.1872	0.3544	<.0001	<.0001	0.0002	0.0002	0.7263
PxG1520			0.907	0.99	0.2732	0.2732	0.4856	<.0001	<.0001	0.0003	0.0003	0.907
PxG1106				0.907	0.3256	0.3256	0.5605	<.0001	<.0001	0.0004	0.0004	0.99
PxG1110					0.2732	0.2732	0.4856	<.0001	<.0001	0.0003	0.0003	0.907
PH-1						0.99	0.6831	<.0001	<.0001	0.0046	0.0046	0.3256
Gz3639GFP							0.6831	<.0001	<.0001	0.0046	0.0046	0.3256
Gz3639								<.0001	<.0001	0.0017	0.0017	0.5605
PxG1517									0.4856	0.0053	0.0053	<.0001
PxG1122										0.0267	0.0267	<.0001
PxG1601											0.99	0.0004
PxG1621												0.0004
PxG1521												



**Figure 3.12B.** Comparison of average DON production *in planta* among parental strains, PH-1 and Gz3639GFP, and transgressive progeny strains.

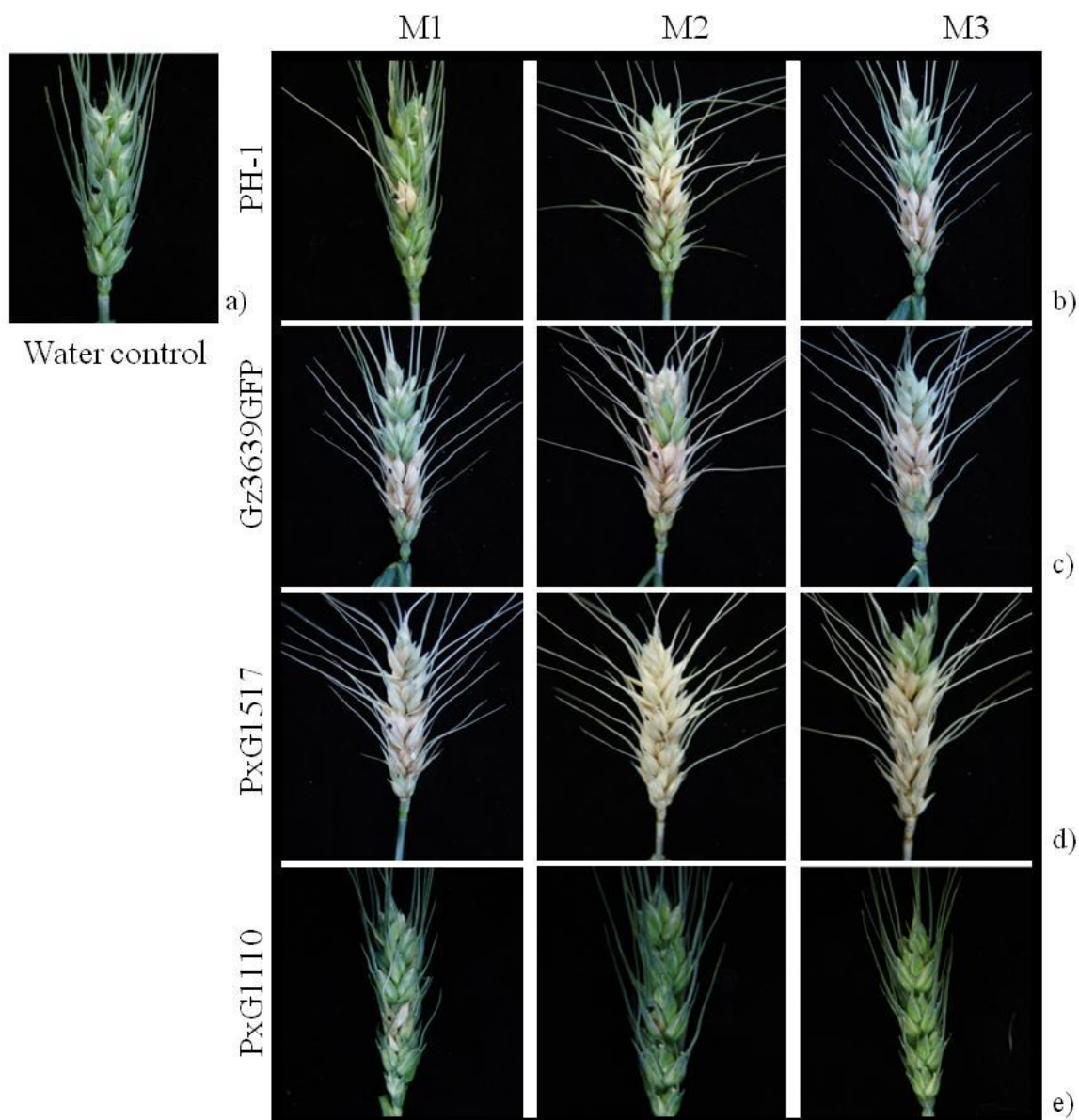
**Table 3.7:** Table: LSD matrix of the pair-wise comparisons for the effect of fungal strain on DON production *in planta*. The values in red indicate statistical difference at the level of  $P \leq 0.05$ .

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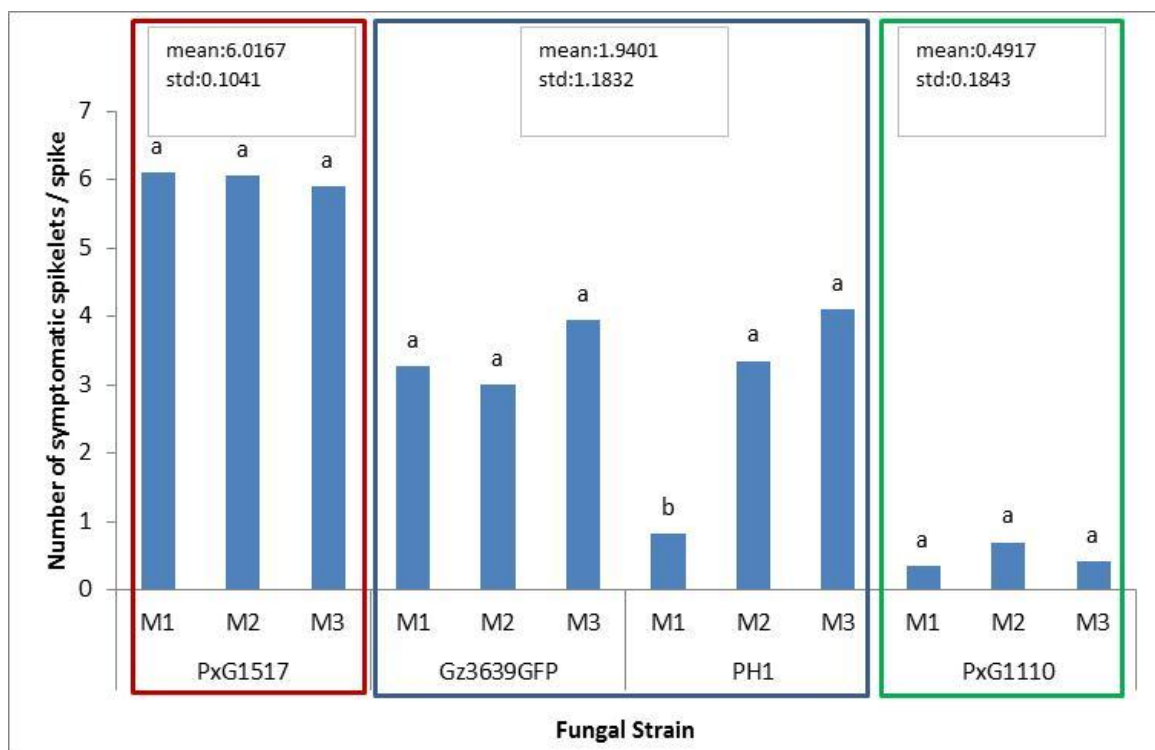


**Figure 3.12C:** Comparison of average DON production in medium (2% GYPD) among parental and transgressive strains.





**Figure 3.13.** Comparison of external FHB severity symptoms produced by mitotic progeny strains from parental strains PH-1 (b), and Gz3639GFP (c), and the transgressive segregant progeny strains PxG1517 (d) and PxG1110 (e) with the water control (a) point-inoculated susceptible wheat variety Pioneer 2555. The inoculated floret is marked with a dot.

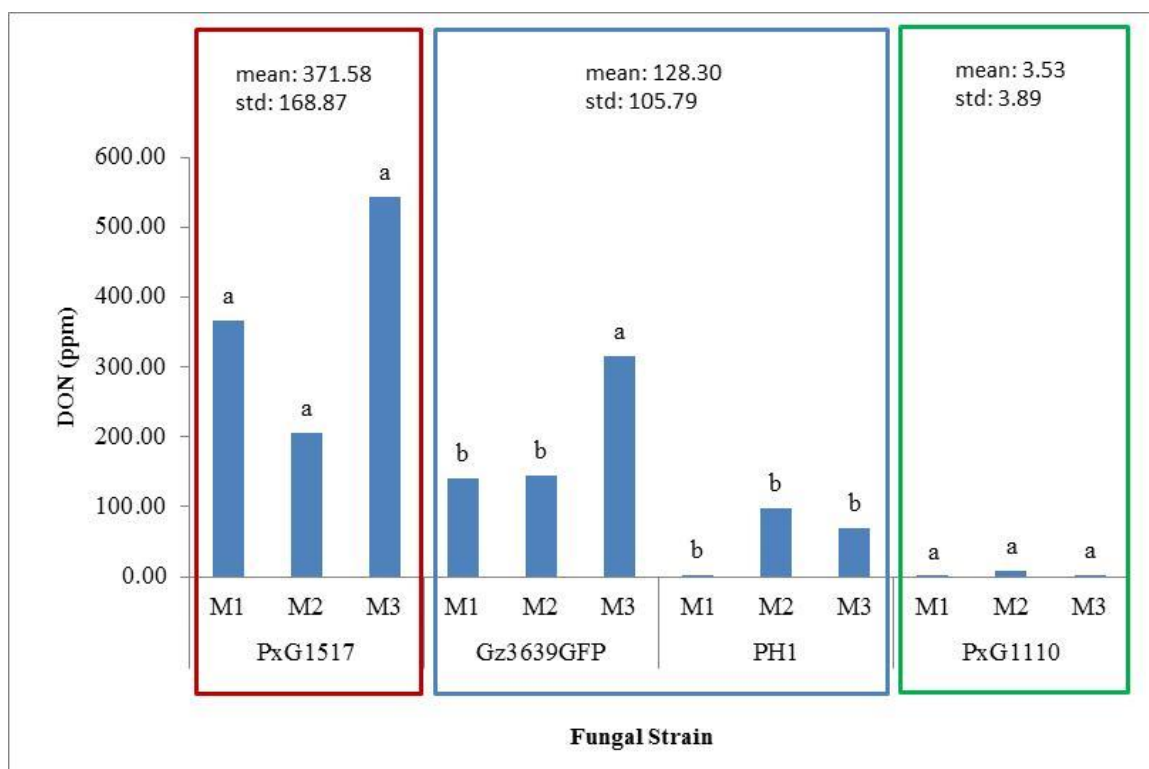


**Figure 3.14A:** Mitotic Stability of the FHB aggressiveness phenotype in the transgressive progeny strains PxG1517 and PxG1110. The letters indicate statistical differences between strains in the boxes at a P-value= 0.05. The red, blue, and green boxes encase three mitotic isolates for positive transgressive segregant (PxG1517), parental strains (Gz3639GFP and PH-1), and negative transgressive segregant (PxG1110), respectively.



**Table 3.9:** LSD matrix of the pair-wise comparisons between the mitotic progenies of parental strains, and positive, and negative transgressive segregating strains testing the effect of fungal strain on FHB aggressiveness (expressed as 7 dpi FHB symptom severity). The values in red indicate statistical difference at the level of  $P \leq 0.05$ .

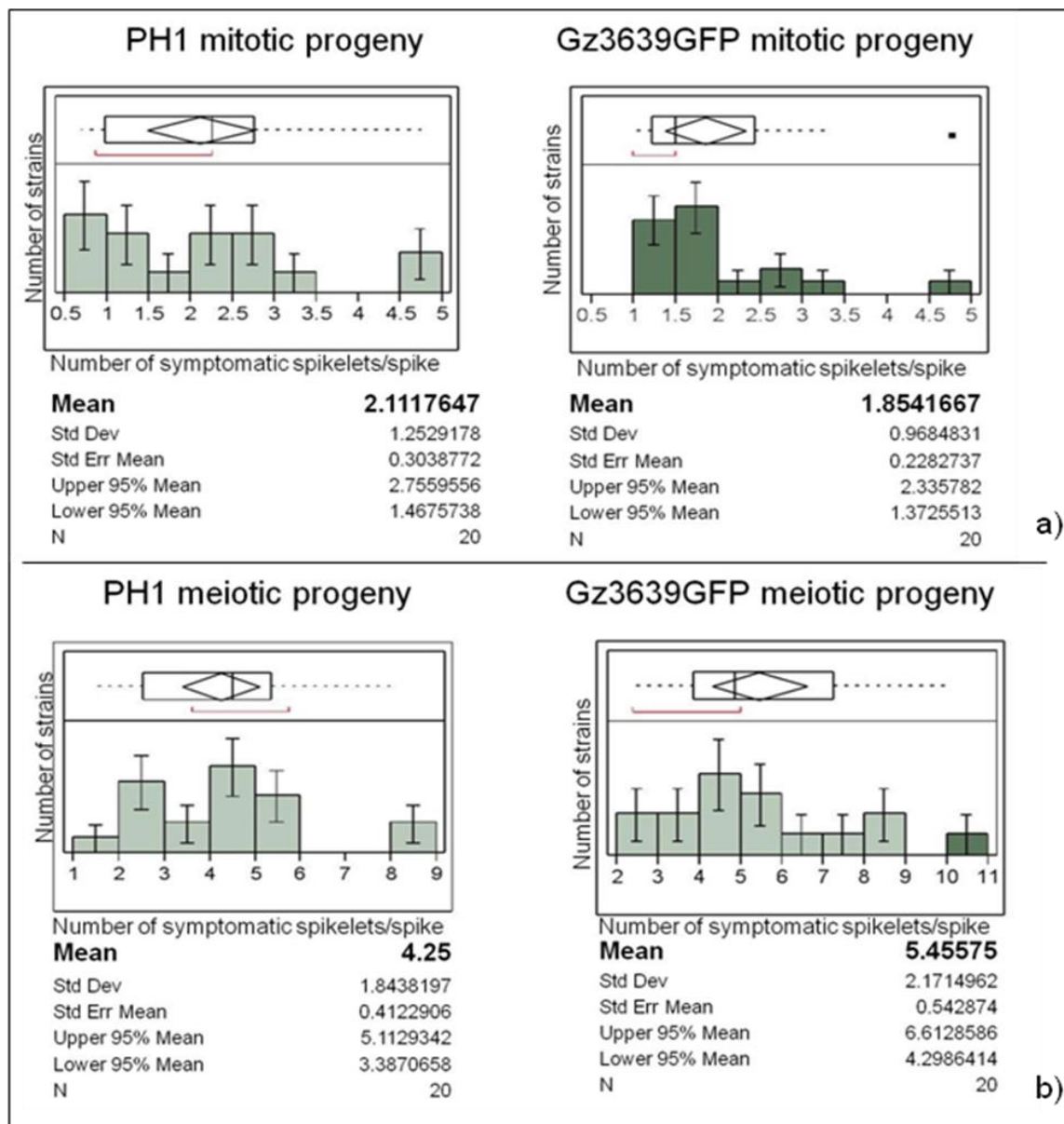
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**Figure 3.14B:** Mitotic Stability of the DON production in the transgressive progeny strains PxG1517 and PxG1110. The letters indicate statistical differences between strains in the boxes at the P-value= 0.05. The red, blue, and green boxes encase three mitotic isolates for positive transgressive segregant (PxG1517), parental strains (Gz3639GFP and PH-1), and negative transgressive segregant (PxG1110), respectively.

**Table 3.10.** LSD matrix of the pair-wise comparisons between the mitotic progenies of parental strains, and positive, and negative transgressive segregating strains testing the effect of fungal strain on DON production *in planta*. The values in red indicate statistical difference at the level of  $P \leq 0.05$ .

[illegible]



**Figure 3.15.** Comparison of the aggressiveness phenotypes between progeny derived through mitosis (asexual) (a), and meiosis (sexual) (b).

## **CHAPTER 4**

### **Relative efficiency of a “split-marker” protocol for the production of deletion mutants in strain PH-1 of *Fusarium graminearum***

#### **4.1 Introduction**

The availability of whole-genome sequences for an increasing number of fungal species has prompted the development of more efficient technologies to utilize these data for functional genomics research (Vukmirovic and Tilghman 2000). Inactivation of a gene by deletion or interruption is an important way to study its function (Shafran et al. 2008). Gene deletion via double-crossover replacement with a selectable marker is usually more desirable than gene disruption, because it avoids the possibility of residual or restored gene function. Numerous polymerase chain reaction (PCR)-based protocols have been developed to facilitate the use of genome data for production of gene deletion constructs (e.g. Hamann et al. 2005; De Hoogt et al. 2000; ML Nielsen et al. 2006; Paz et al. 2011; Ray and Adholeya 2008; Shafran et al. 2008; Turgeon et al. 2010; Yu et al. 2004). Deletion constructs typically consist of varying amounts of sequence from the 5' and 3' flanks of the gene of interest fused to a selectable marker gene, and are referred to here as “intact-marker” (IM) replacement constructs.

Successful gene deletion is limited by the efficiency of homologous recombination (Hynes 1996a). Non-homologous end joining (NHEJ) and homologous end joining (HEJ) are two ways in which fungi repair double-stranded breaks in their DNA (Cahill et al. 2006; Kanaar et al. 1998). The yeast *Saccharomyces cerevisiae* primarily uses HEJ, and generation of gene deletion mutants is very efficient in this organism (Hua et al. 1997). In contrast, a majority of filamentous fungi use the NHEJ mechanism for DNA repair, and homologous recombination occurs only rarely (with a frequency of less than 1%) (Kück and Hoff, 2010). This results in relatively few transformants in which the gene of interest has been replaced by a selectable marker, and more strains with the marker integrated at an ectopic location in the genome (Chaveroche et al. 2000). To increase the frequency of homologous recombination events, some researchers have used mutant

strains deficient in NHEJ as transformation recipients (De Boer et al. 2010; Carvalho et al. 2010; Catalano et al. 2011; Li et al. 2010; Pöggeler and Kück 2006; Snoek et al. 2009). However, these strains have an elevated sensitivity to various mutagens, so transformants must usually be back-crossed to a wild-type strain before they can be used for experiments (Kück and Hoff, 2010).

The split-marker (SM) protocol, first applied to yeast (Fairhead et al., 1996), is an alternative approach for increasing the relative frequency of homologous integration in wild-type strains of filamentous fungi (Catlett et al. 2003; De Hoogt et al. 2000; You et al. 2009). For this procedure, fungal protoplasts are transformed with a mixture of two DNA fragments, comprised of DNA flanking each end of the gene of interest fused to overlapping segments of a selectable marker gene. In order to reconstitute a functional selectable marker, the SM fragments must recombine homologously at three points, which hypothetically increases the probability of a gene replacement versus an ectopic integration among the resulting selectable transformants. However, this hypothesis has been tested experimentally in very few cases (e.g. Fu et al. 2006; You et al. 2009). The SM protocol has been recommended for production of gene deletions in *Fusarium graminearum*, an important fungal pathogen that causes head blight disease on wheat (Catlett et al. 2003). The objective of the current study was to compare SM and IM protocols, and evaluate their relative efficiency for generating gene deletion mutant strains and appropriate control strains with ectopic integrations, in strain PH-1 of *F. graminearum*, which has an available high-quality (> 8X) annotated genome sequence. For this work, the well-defined mating-type locus of *F. graminearum*, which has a previously characterized deletion phenotype (Lee et al. 2003) was targeted.

## 4.2. Materials and Methods

**Fungal strains and growth conditions:** *Fusarium graminearum* strain PH-1 (NRRL 31084) was used for all experiments. The cultures were stored as a silica stock (Tuite, 1969, after Perkins, 1962), and were never sub-cultured more than once. For production of asexual spores (macroconidia), the fungus was cultured on mungbean agar (MBA) (Bai and Shaner 1996) for 7-10 days at 23° C under continuous fluorescent light

(Sylvania F032/741/ECO). Spore suspensions were prepared by adding 2 ml of sterile water to the culture and gently rubbing the surface with a sterile plastic micro-pestle. Harvested spores were filtered through glass wool, washed twice with sterile water, and adjusted to the desired concentration.

**DNA extraction:** For DNA extraction, 5 ml of YEPD medium (20 g dextrose, 20 g bacto-peptone, 10 g yeast extract) was inoculated with an 8-mm agar plug taken from the edge of an actively growing colony. Cultures were incubated at 25° C for 5-7 days at 250 rpm. Mycelia were harvested by decanting cultures onto sterile paper and blotting the excess liquid. Mycelia were flash-frozen in liquid nitrogen and stored at -80° C until extraction.

Frozen mycelia were lyophilized and then pulverized in individual 2 ml Eppendorf tubes by using a mini-pestle, or in 96-deep well plates by using a 2000 GENO Grinder (Spex Cretiprep) (500 strokes/sec for 30 sec). One ml of warm lysis buffer (0.5 M NaCl, 1% SDS, 10 mM Tris HCl, Ph7.5, 10 mM EDTA) was added per 100-200 mg of pulverized fungal tissue, and the samples were incubated at 65° C for 30 min, vortexing once during the incubation. After incubation the samples were transferred into individual tubes containing 660 µl PCI (25 parts phenol, 25 parts chloroform, 1 part iso-amyl-alcohol), mixed by inverting 4-6 times, then incubated at 65° C for an additional 30 min. The contents were mixed once again during incubation. The samples were centrifuged in a tabletop centrifuge for 20 min at maximum speed to separate the phases. DNA was precipitated from the aqueous phase by using 1 volume of isopropanol, and the pellet was washed twice with 70% ethanol. The pellet was resuspended in 100 µl of TE, pH 7.9, and 2 µl of a 5-mg/ml concentration of RNase A, at 65° C for 1 h. Between 1 and 5 µg of each DNA sample was digested with appropriate restriction enzymes (RE) and used in Southern hybridizations (Southern, 1975) for evaluation of transformant and wild-type strains.

**Plasmid Construction:** The strategy for construction of plasmid templates and generation of SM and IM replacement fragments is shown in Figure 4.1. For each of the genes of interest, 0.6-1.2 kb of each flank was amplified. The PCR primers are listed in Table 4.1, and the PCR parameters used are shown in Table 4.2. The primers were designed manually from the published *F. graminearum* genome (Cuomo et al. 2005). Primer P2 incorporated a 3' *EcoRI* recognition site, and primer P3 included a 5' *BamHI* recognition sequence. These same restriction enzymes (REs) were used to isolate the hygromycin phosphotransferase (*hph*) selectable marker gene from the donor plasmid pBSHyg2 (pBluescript plasmid was digested with *SmaI* and *hph* resistance gene blunt ligated to form pBSHyg2).

PCR amplicons and the restricted *hph* gene fragment were gel-purified with a gel extraction kit (QIAquick Gel Extraction Kit, Qiagen). PCR amplicons were digested with the appropriate RE and then gel-purified once more. The restricted fragments were ligated in a 1:2:1 molar ratio with T4 ligase (Invitrogen) at 16° C overnight. The ligase was inactivated by incubating the reactions at 70° C for 20 min. The reactions were diluted 1000-fold and used as PCR templates with primers P1 and P4 to produce the intact-marker cassette amplicons.

IM cassette amplicons were gel-purified and cloned into the pGEM T-easy plasmid. Before cloning, “A-tails” were added by incubating 15 µl of each gel-purified amplicon with 0.4 µl Taq polymerase, 2 µl 10x PCR buffer, 0.6 µl 50 mM MgCl<sub>2</sub> and 2 µl of 10 mM dATP for 30 min at 72° C. The plasmids were introduced into electro-competent DH5α *E. coli* cells, and clones were confirmed by restriction digestion and sequencing, then stored as 15% glycerol stocks at -80° C.

**PCR amplification of split-marker fragments, and intact-marker replacement cassettes:** The plasmids were used as templates for generation of SM fragments and IM replacement cassettes (Figure 4.1). PCR reactions consisted of a 25 µl total reaction volume including 2.5 µl of 10X Phusion PCR buffer, 2.5 µl of 25 mM MgCl<sub>2</sub>, 2 µl of 10 mM dNTP mix, 1 µl of each primer (20 nM) and 1 µl of template DNA (20-50 ng/µl).



The thermocycling parameters were as follows: initial denaturation for 3 min at 94° C, followed by 35 cycles of denaturation at 94° C for 30 sec, annealing at 60° C for 20 sec, and extension at 72° C for 1 min/kb, with a final extension for 7 min at 72° C. The PCR amplicons were gel-purified and eluted in 15 µl sterile water to yield 1-3 µg DNA.

**Preparation of fungal protoplasts:** Protoplast isolation and transformation protocols were modified from methods used for transformation of *Aspergillus parasiticus* (Skory et al. 1990; F. Trail, personal communication). Macroconidia were harvested from 7 day-old cultures, washed, and resuspended at a concentration of  $1 \times 10^6$  spores/ml. 100 ml of YEPD was inoculated with 2 ml of the spore solution. The culture was incubated for 10-14 hours at 25° C with constant agitation (175 rpm). Mycelia were harvested by filtration, washed with sterile water, and treated with 20 ml of protoplasting buffer [500 mg driselase (Sigma Chemical Co., St. Louis); 100 mg lysing enzyme from *Trichoderma harzianum* L-1412 (Sigma Chemical Co., St. Louis); in 20 ml of 1.2 M KCl, filter sterilized]. Mycelium was incubated in the protoplasting buffer for 45-90 minutes at 37° C with gentle agitation (50-80 rpm). When the majority of hyphae had released protoplasts, the solution was filtered through a 30 µm Nitex nylon membrane into sterile 50 ml centrifuge tubes. Protoplasts were pelleted by centrifugation (3000 rpm) at room temperature for 5 min, then gently resuspended in STC buffer (1.2 M sorbitol; 10 mM Tris-HCl, pH 8.0; 50 mM CaCl<sub>2</sub>). Protoplasts were pelleted once more, and then they were resuspended in STC at a concentration of  $1 \times 10^8$  protoplasts/ml.

**Transformation of *Fusarium graminearum*:** For each transformation, 100 µl of freshly prepared protoplast suspension ( $1 \times 10^8$  protoplast/ml) was mixed with 100 µl STC buffer, 50 µl freshly prepared and filter-sterilized 30% PEG solution (8000 polyethylene glycol, Sigma, P2139) dissolved in a buffer (10 mM Tris-HCl, pH 8.0; 50 mM CaCl<sub>2</sub>) and 10 µl of DNA (1-3 µg). For SM fragments, 6 µl of each fragment was mixed together first, and then 10 µl of the mixture was added to the transformation. The reaction was incubated at room temperature for 20 min. Two ml of 30% PEG Solution was added, and incubation

continued for an additional 5 min. Four ml of STC buffer was added, and gently mixed by inversion. The transformation reaction was then added to 250 ml of cooled regeneration medium (RM) (1.0 g yeast extract; 1.0 g N-Z-Amine AS (N4517 Sigma); 7.44 g agar; and 271 g sucrose per liter). The medium was mixed gently and aliquoted into three 100 mm Petri plates. Protoplasts were allowed to regenerate for 12-15 hours, and then the plates were overlaid with 15 ml of RM amended with 150 µg/ml hygromycin B. Transformants usually appeared within 4-7 days, when they were transferred to potato dextrose agar (Difco<sup>®</sup>) containing 450 µg/ml hygromycin B.

**Analysis of transformants:** Transformants were single-spored, and genomic DNA extracted from each strain was evaluated by using Southern hybridization analysis to characterize integration events. The fertility phenotypes of confirmed gene deletion mutants and ectopic controls were assessed by using the mycelial plug crossing method (Bowden and Leslie 1999). Briefly, 5 mm diameter plugs taken from the edges of actively growing colonies of the parental strains were placed on opposite halves of a 60 mm diameter Petri plate containing carrot agar (400 g fresh, peeled and diced carrots were autoclaved in 400 ml H<sub>2</sub>O for 20 min and homogenized with a blender. After homogenization the volume was adjusted to 1 L and 20 g of Difco<sup>®</sup> agar was added to the slurry. The media was autoclaved for additional 30 min, and poured into 60 mm Petri plates (Leslie and Summerell, 2006). The colonies were incubated for 4 days at 23° C with constant fluorescent light. When the colonies met in the middle of the plate, 0.5-1.0 ml of 2.5% Tween 60 was applied to the surface of the plate, and the mycelia were flattened by rubbing gently with a sterile glass rod to induce perithecial production. Presence and appearance of perithecia produced on the crossing plates, and presence of asci containing ascospores, were assessed 18 days post-induction. The segregation ratio of MAT1-1-1 and MAT1-2-1 specific markers among the progeny derived from a cross of a  $\Delta$ mat1-1-1 x a  $\Delta$ mat1-2-1 deletion strain was determined by Southern blot analysis.

### 4.3. Results

#### Comparison of SM and IM approaches

The mating type locus of *F. graminearum*, known as MAT1, is a complex locus that contains four open reading frames. Two of these (MAT1-1-1 and MAT1-2-1) encode homeodomain proteins that interact to switch on mating-dependent development (Kronstad and Staben 1997). In this study, three sequences incorporating *F. graminearum* mating genes were targeted for deletion. These included the entire MAT1 locus (6070 bp), the MAT1-1-1 gene (1117 bp), and the MAT1-2-1 gene (978 bp). SM and IM constructs were produced for each sequence and used in transformation experiments. Each experiment generated between 50 and 70 hygromycin-resistant transformants within 4-7 days, from which 20 - 25 individual transformants were randomly chosen for further analysis. There were no noticeable differences among the different constructs in the number or appearance on RM of the transformants they generated.

Southern hybridization analysis revealed the presence of three different types of integrations among the recovered transformants, including double-point homologous recombination (HR) resulting in gene deletion; ectopic integration by non-homologous end joining (NHEJ); and a mixture of the two, consisting of a single point homologous recombination combined with non-homologous end joining (HR:NHEJ) (Figure 4.2a and 4.2b). This last type of integration did not result in a gene deletion, but instead in the integration of the disruption construct directly adjacent to the target gene.

Both types of constructs (SM and IM) generated gene deletion strains. The gene deletion rate ranged from 25-75%. The SM constructs appeared to be somewhat more efficient in generating deletion strains than the IM constructs for the large MAT1 locus, but the differences were less noticeable for the smaller MAT1-1-1, and IM constructs actually produced slightly more deletions of the smallest MAT1-2-1 fragment (Table 4.4). Among the strains with HR events resulting in gene deletions, approximately one-third had additional ectopic integrations of the DNA. Both approaches generated a similar proportion of strains with additional ectopic integrations (27% for SM, 30% for IM). In comparison with IM, SM generated very few transformants with single ectopic integrations. The SM produced only one transformant strain with a single ectopically

integrated copy of the MAT1-2-1 gene replacement construct sequence, while IM generated 17 such strains (Table 4.4), representing all three sequences. SM produced many more strains with HR:NHEJ combined events. In fact, SM produced many more NHEJ events overall than IM. Eighteen percent of the NHEJ integration events were integrations of just one of the SM flanks: of those single flank integrations, 74% were of the 5' flank. Transformation experiments in which the IM constructs were linearized by cutting within the plasmid sequence, thus avoiding the creation of homologous recombinogenic ends, produced similar numbers of HR and NHEJ integration events (Figure 4.4).

There was not a strong relationship between GC% of flanking regions and the efficiency of HR, however both approaches generated slightly more strains with a single HR integration when the targeted sequence had higher GC content (Table 4.5).

**Phenotypic characterization of the mutant strains:** Targeted deletion of the MAT genes by either SM or IM resulted in the expected phenotypes, and there were no apparent differences related to the deletion approach used. As has been reported previously, MAT1 deletion strains produced small perithecial initials containing no ascospores after induction (Desjardins et al., 2004). MAT1 deletion mutants were completely infertile, and were able to reproduce only asexually. Strains from MAT1 deletion experiments with single ectopic integrations of the MAT1 sequence had the normal wild type (WT) fertility phenotype. The perithecia were of normal size and contained ascospores.

Deletion of either MAT1-1-1 or MAT1-2-1 resulted in the loss of self-fertility. After induction,  $\Delta$ mat1-1-1 and  $\Delta$ mat1-2-1 strains produced small perithecial initials containing no ascospores, similar to the MAT1 deletion strains. The single-copy ectopic strains from MAT1-1-1 and MAT1-2-1 deletion experiments retained the WT fertility phenotype. As reported previously (Lee et al. 2003),  $\Delta$ mat1-1-1 and  $\Delta$ mat1-2-1 strains behaved as obligate heterothallic strains. A cross of  $\Delta$ mat1-1-1 x  $\Delta$ mat1-2-1 resulted in normal sized fertile perithecia containing asci with eight ascospores. MAT1-1-1 and MAT1-2-1 gene-specific markers showed normal 1:1 segregation among progeny from the  $\Delta$ mat1-1-1 x  $\Delta$ mat1-2-1 cross (Figure 4.5).

#### 4.4. Discussion

Targeted gene deletion (“gene knockout”, KO) is commonly employed as a strategy to understand gene function (Weld et al. 2006). However, it is usually difficult to create targeted deletions in filamentous fungi because the rate of homologous recombination (HR) is relatively low (Hamann et al. 2005; Hynes 1996b; Ruiz-Díez 2002). Many factors have been reported to influence the frequency of HR events, including: transformation protocol (e.g. PEG-mediated, *Agrobacterium*-mediated); fungal species; length and degree of homology between the deletion construct and the targeted sequence; length of the targeted sequence; position of the gene in the genome; and the GC content of the targeted sequence (Catlett et al. 2003; Collopy et al. 2010; Gray and Honigberg 2001; Hynes 1996b; Idnurm et al. 2004; L Hamer et al. 2001; Meyer et al. 2003; Mullins and Kang, 2001). Various strategies have been employed to increase the frequency of HR and thus of gene KO. In this study I have evaluated the relative efficiency of one of these strategies, the split-marker (SM) protocol, by comparing it with a more standard intact marker (IM) gene replacement protocol. In theory, the SM protocol requires HR to occur at three points in order to obtain a viable transformant, instead of at only two points as in the IM protocol. Because of this difference, use of the SM strategy would be expected to increase the frequency of HR among surviving transformants (Catlett et al. 2003; Fu et al. 2006).

I found that both strategies were similar in their ability to produce KO strains via HR. Both protocols produced KO mutants with the expected phenotypes. Neither protocol produced a majority of KO strains; both produced large numbers of transformants with ectopic integrations. There were no major differences for any of the three genes I tested between the SM and IM protocols, although the SM seemed to be slightly more efficient for KO of the longest sequence, the MAT1 locus. However, the numbers of transformants and the differences were too small for me to conclude that SM will always be more appropriate for KO of longer sequences. Given that SM requires one more step in the protocol than IM, there appears to be little advantage in using it for gene KO in PH-1 if the goal is to increase KO efficiency. In fact, the extra step to produce the linear IM construct may not even be necessary, since just linearizing the template plasmid and

using that in the transformation produced results that were similar to those obtained with the PCR product. Theory suggests that SM constructs integrate separately into the genome and then, only if they are close enough, they recombine a third time to regenerate the functional selectable marker. However, it appears that in a substantial number of cases, the SM constructs may recombine within the selectable marker prior to integration into the genome, resulting in transformants that are resistant to the selection but don't have a KO. Heterologous integration appears to be quite efficient in PH-1.

One big difference I observed between the two protocols was related to the number of ectopic integrations. The SM protocol produced many more independent ectopic integrations, and more integrations per strain, than the IM protocol. Many of the ectopic integrations were of single SM constructs. The increased number of ectopic integrations with the SM protocol could relate to the increased number of recombinogenic ends per mole for the SM constructs, when they don't recombine prior to integrating. Ectopic integrations are used to create random mutations, for example with the restriction enzyme mediated integration (REMI) protocol (Mullins and Kang, 2001). It is important, when choosing strains for KO studies, to avoid any that contain additional ectopic insertions since these can cause independent mutations. Additional mutant phenotypes will make the results of the experiment difficult to interpret (Brown and Holden 1998). Since IM produced more KO strains without extra ectopic integrations, IM would be preferred for production of KO strains in PH-1.

It is important to use appropriate controls in a KO experiment. The most useful controls are strains that have a single, unlinked (NHEJ) ectopic integration of the deletion construct. As above, strains with multiple ectopic integrations are less useful as they may have multiple phenotypes that could make results of the experiment difficult to interpret. Strains with HR-NHEJ integrations are also not useful as controls since the integration disturbs the neighborhood of the target gene and may have effects on its expression. An appropriate ectopic control ensures that an observed phenotype is related to the deletion of targeted sequence and not to the presence of the deletion cassette in the genome. The SM protocol generated very few suitable ectopic control strains. Thus, once again, the IM protocol would be preferred to the SM approach.

## 4.5. References

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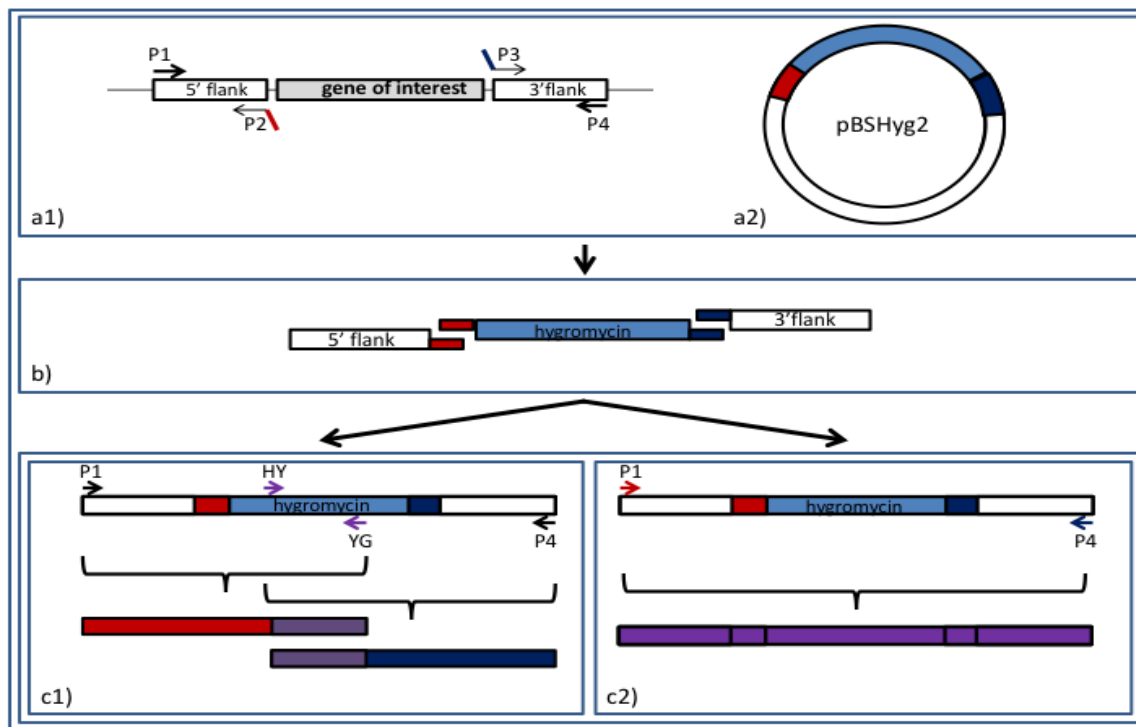


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**Table 4.1.** List of primers used in comparison of split-marker (SM) and intact marker (IM) approach to targeted gene deletion.

Gene of Interest	Primer	Amplicon	Primer sequence 5'→3'
MAT1	GzMAT1P1	MAT1 5' flank forward	GCGCTTATATCGGGCATAGA
	GzMAT1P2 <i>EcoRI</i>	MAT1 5'flank reverse + <i>EcoRI</i> site	AAAGGAATTCGGCGTTCTGAGAGTTGGA
	GzMAT1P3 <i>BamHI</i>	MAT1 3'flank forward + <i>BamHI</i> site	AAAAGGATCCTGCATTGATTTGAGCCAG
	GzMATP4	MAT1 3'flank reverse	TCTCACAACGGCAACTGTTC
MAT1-1-1	GzMAT111F	MAT111 internal probe forward	AGTCCGAATGAAGCCCCAATACC
	GzMAT111R	MAT111 internal probe reverse	CAGAACTTGCAGGTGCTGGGAGT
	GzMAT111P1	MAT1 5' flank forward	GGCCGATAATCTCCTCGACT
	GzMAT111P2 <i>EcoRI</i>	MAT1 5'flank reverse + <i>EcoRI</i> site	AAGCGAATTCGCACGGAATCGTTCCAGA
MAT1-2-1	GzMAT111P3 <i>BamHI</i>	MAT1 3'flank forward + <i>BamHI</i> site	AAGGGGATCCTTAAATTGCAGAGGTGTGTAAGG
	GzMAT111P4	MAT1 3'flank reverse	TCTATGTTAGTAGGCAGCAGTGG
	GzMAT121F	MAT121 internal probe forward	TCTTCCACCCCCTGTGTCTACCA
	GzMAT121R	MAT121 internal probe reverse	TGCGAATGTCAGGATGCTCCA
MAT1-2-1	GzMAT121P1	MAT121 5' flank forward	GGCATAGAGTCGTCCCAGAA
	GzMAT121P2 <i>BamHI</i>	MAT121 5'flank reverse + <i>BamHI</i> site	GTAAAGGATCCTCAGATGAAGTTGGCAGGTG
	GzMAT121P3 <i>EcoRI</i>	MAT121 3'flank forward + <i>EcoRI</i> site	TCGAGAAATTCCTCAGGCCCTACGTTTTGTT
	GzMAT121P4	MAT121 3'flank reverse	CCTGCAAGTTCTGATGTGGA
Hyg. marker cassette	HY	Internal hygromycin probe forward	GGATGCCTCCGCTCGAAGTA
	YG	Internal hygromycin probe reverse	CGTTGCAAGACCTGCCTGAA



**Figure 4.1.** Schematic depiction of split-marker and intact-marker gene-replacement strategies.

a1) PCR amplification of gene flanking regions with overhangs with RE recognition sequences; a2) release of hygromycin resistance gene from pBSHyg2 by digestion with *EcoRI* and *BamHI*; b) Ligation of PCR amplified gene flanking regions with digested ends and hygromycin gene released from pBSHyg2; c1) PCR amplification of split-marker fragments with combination of flanking region (5' flankP1 or 3' flankP4 primer and hygromycin gene specific primer (HY or YG)); c2) PCR amplification of an intact marker cassette with gene specific 5' flankP1 and 3' flankP4 primers

**Table 4.2.** Summary of the PCR thermocycling protocols for amplification of gene specific flanking regions for four genes used in comparison of SM and IM gene deletion strategies.

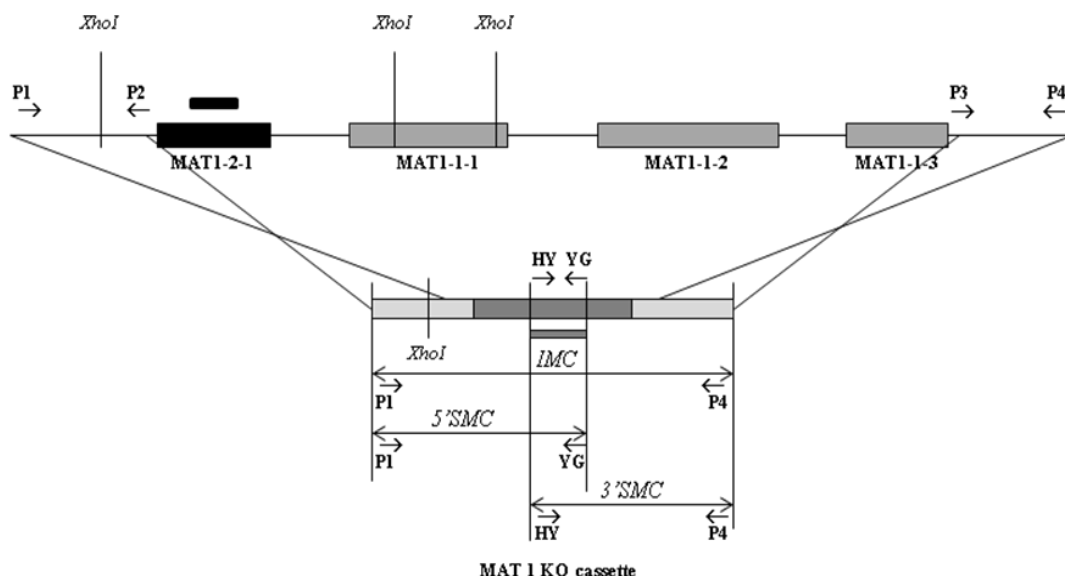
Gene of interest	Flanking region	Primer set	PCR thermocycling protocol					
			Initial denaturation	PCR amplification			Final extension	
				Denaturation	Annealing	Elongation		
MAT 1	5' flank	GzMAT1P1 / GzMAT1P2EcoRI	94	94	60	72	72	temp. (°C)
			3:00	0:30	0:20	1:00	7:00	time (min)
			1	40			1	# of cycles (x)
	3' flank	GzMAT1P3BamHI / GzMAT1P4	94	94	58	72	72	temp. (°C)
			3:00	0:30	0:20	1:00	7:00	time (min)
			1	40			1	# of cycles (x)
MAT 1-1-1	5' flank	GzMAT111P1 / GzMAT111P2EcoRI	94	94	58	72	72	temp. (°C)
			3:00	0:30	0:20	0:35	7:00	time (min)
			1	40			1	# of cycles (x)
	3' flank	GzMAT111P3BamHI / GzMAT111P4	94	94	60	72	72	temp. (°C)
			3:00	0:30	0:20	0:35	7:00	time (min)
			1	40			1	# of cycles (x)
MAT 1-2-1	5' flank	GzMAT121P1 / GzMAT121P2BamHI	94	94	60	72	72	temp. (°C)
			3:00	0:30	0:20	0:50	7:00	time (min)
			1	40			1	# of cycles (x)
	3' flank	GzMAT121P3EcoRI / GzMAT121P4	94	94	62	72	72	temp. (°C)
			3:00	0:30	0:20	1:00	7:00	time (min)
			1	40			1	# of cycles (x)

**Table 4.3.** List of PCR amplified fragments, PCR primer combination and amplicons sizes used in comparison of split-marker and intact marker cassette gene deletion strategies.

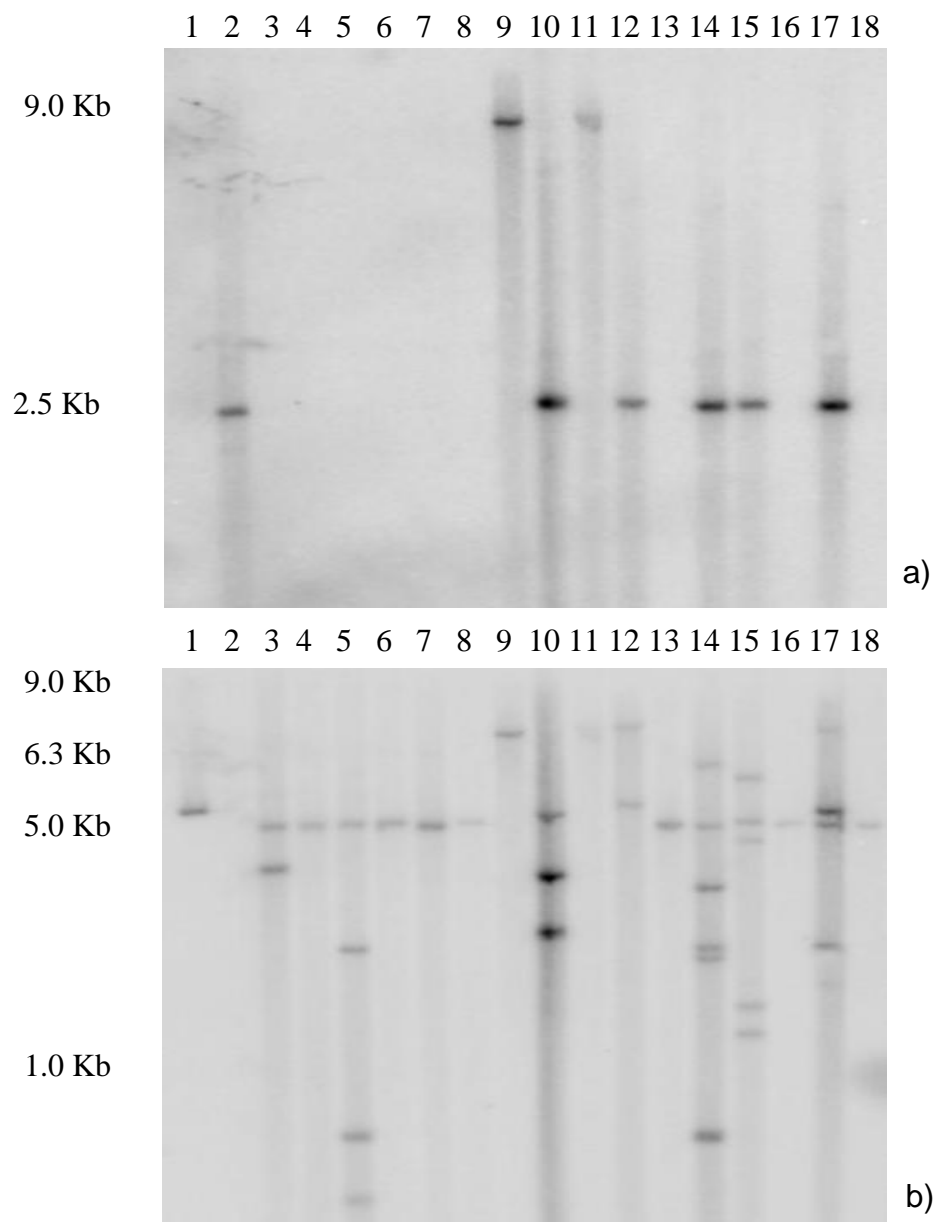
Gene	Gene replacement strategy	Amplicon	Primer set	Amplicon size (bp)
MAT1	SMC*	5' MAT1 SMC fragment	GzMAT1P1 / HY	1702
	SMC	3' MAT1 SMC fragment	YG / GzMAT1P4	2036
	IMC**	MAT1 IMC	GzMAT1 / GzMAT1P4	3272
MAT1-1-1	SMC	5' MAT1-1-1 SMC fragment	GzMAT111P1 / HY	1682
	SMC	3' MAT1-1-1 SMC fragment	YG / GzMAT111P4	1363
	IMC	MAT1-1-1 IMC	GzMAT111P1 / GzMAT111P4	2579
MAT1-2-1	SMC	5' MAT1-2-1 SMC fragment	GzMAT121P1 / YG	1722
	SMC	3' MAT1-2-1 SMC fragment	HY / GzMAT121P4	2118
	IMC	MAT1-2-1 IMC	GzMAT121P1 / GzMAT121P4	3374

\* SMC - split marker cassette

\*\* IMC - intact marker cassette

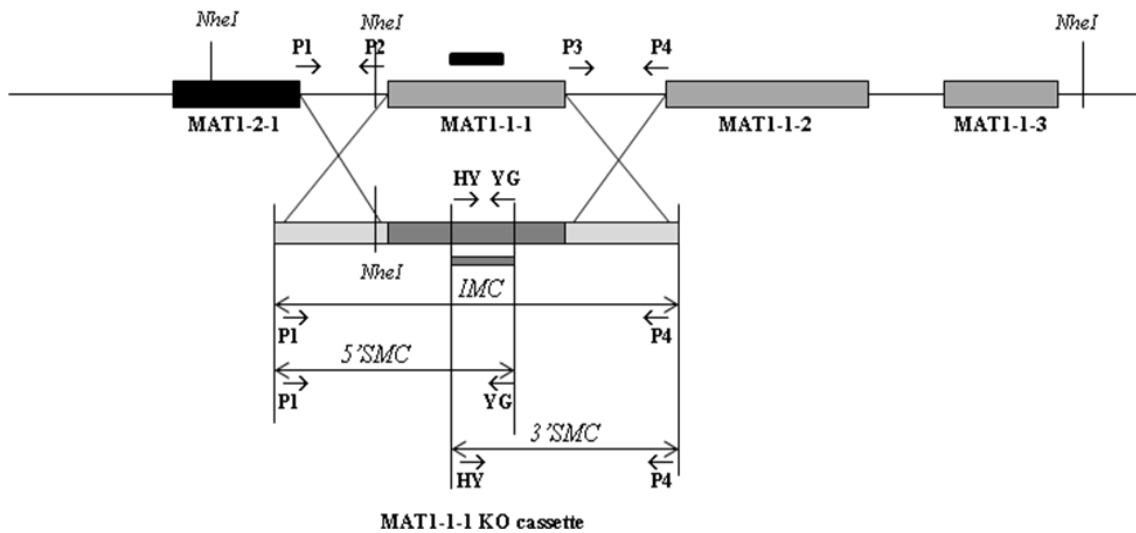


**Figure 4.2A.** Southern blot strategy used to determine types of DNA integration in the fungal strains transformed with MAT1 KO cassette. The genomic DNA from transformant strains have been digested with *XhoI* and probed with MAT1-2-1 specific probe (black bar) and *hph* gene specific probe (gray bar).

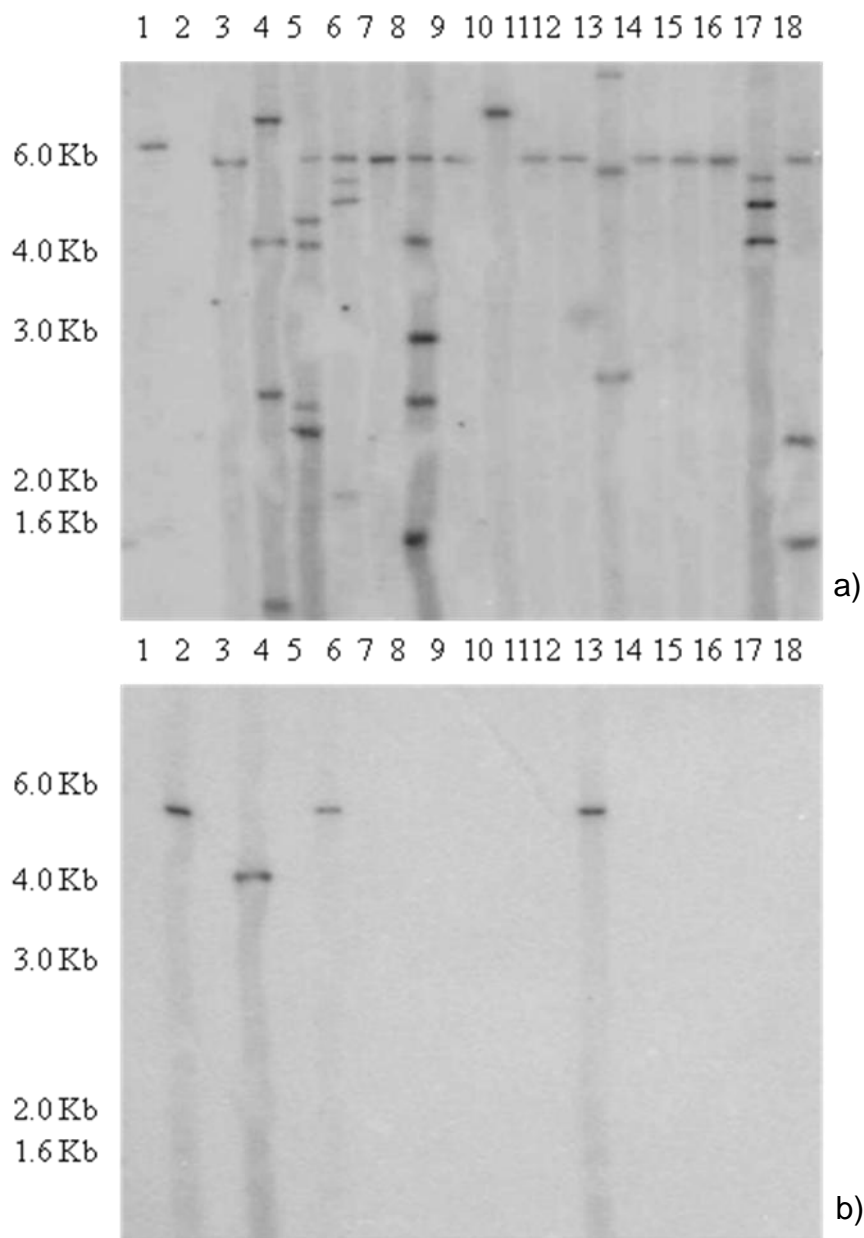


**Figure 4.2B.** Example of types of DNA integrations generated through targeted gene deletion of MAT1 locus: homologous recombination (HR) (lanes 4, 6, 7, 8, 13, 16), ectopic integration (NHEJ) (lanes 12, 14, 15, 17), single point recombination (HR + NHEJ) (lanes 9, and 11). The genomic DNA from the set of  $\Delta$ mat1 transformants was digested with *XhoI* and probed with (a) MAT1-2-1 specific, and (b) hygromycin specific probe.

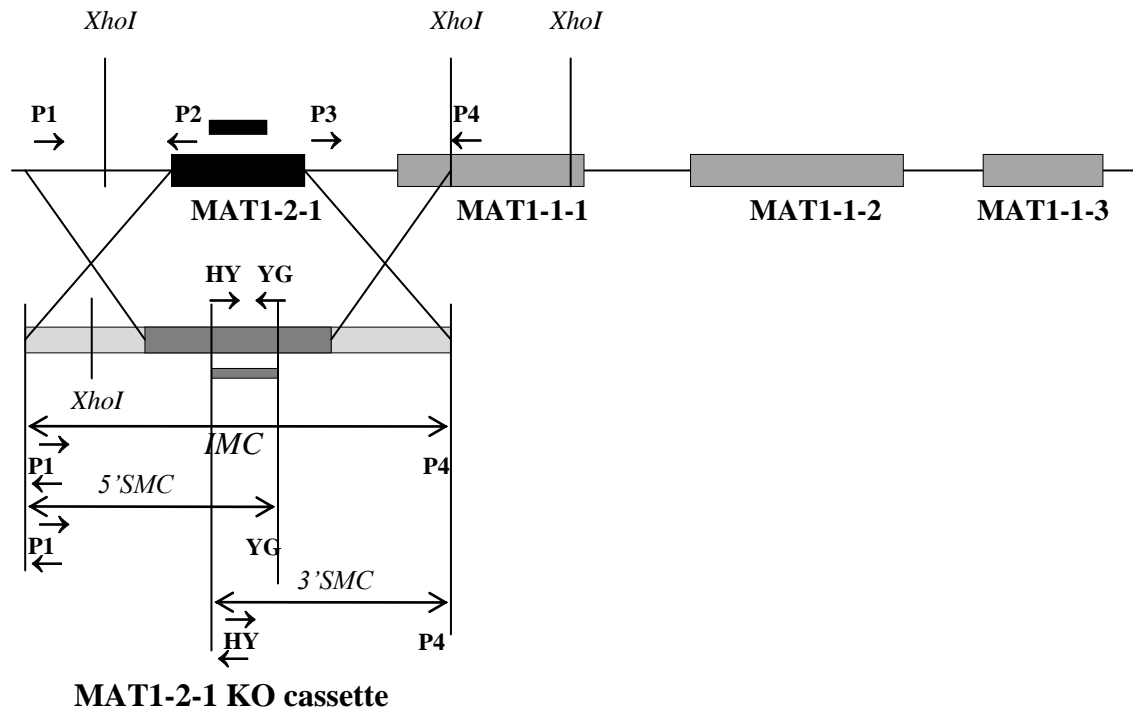




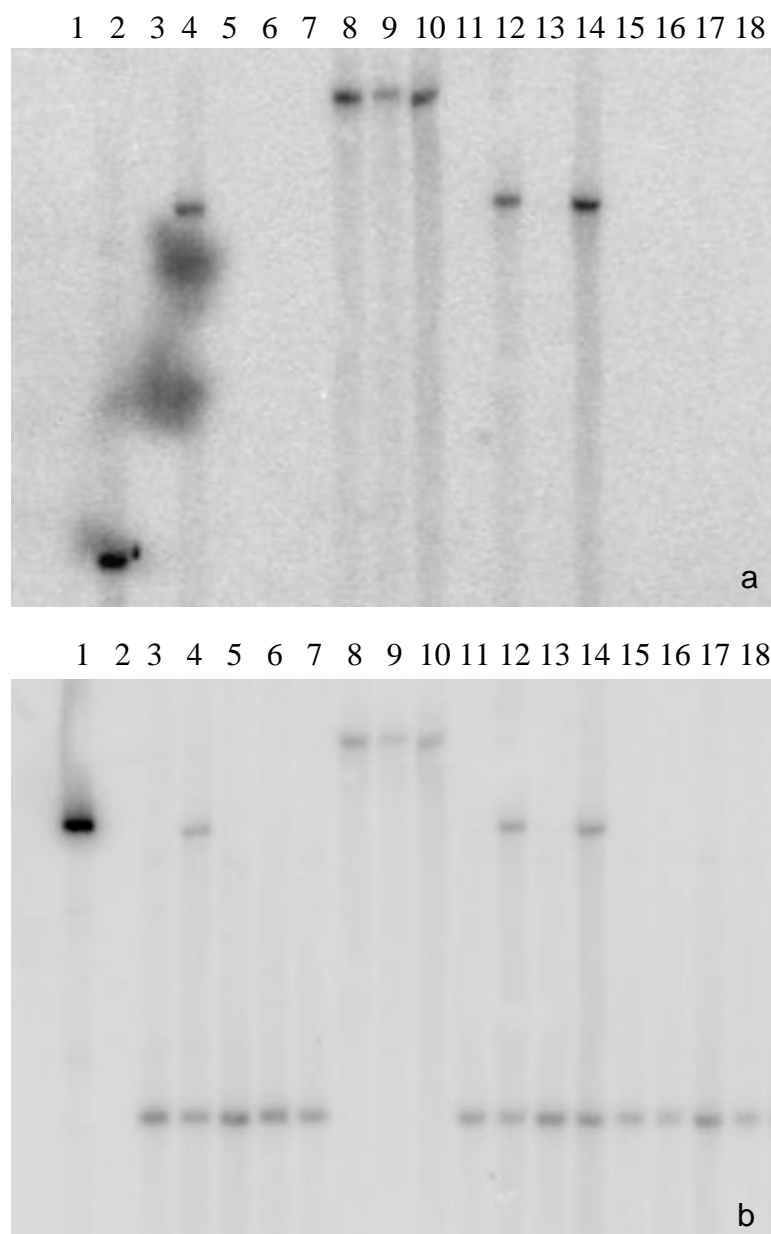
**Figure 4.2C.** Southern blot strategy used to determine types of DNA integration in the fungal strains transformed with MAT1-1-1 KO cassette. The genomic DNA from the set of  $\Delta$ mat 1-1-1 transformant strains have been digested with *NheI* and probed with MAT1-1-1 specific probe (black bar) and *hph* gene specific probe (gray bar).



**Figure 4.2D.** Example of Southern blot analysis used to identify the types of DNA integrations generated through targeted gene deletion of MAT1-1-1 gene: homologous recombination (HR) (lanes 3, 5, 7, 8, 9, 10, 11, 12, 14, 15, 16, 17, 18), ectopic integration (NHEJ) (lanes 6, 13), single point recombination (HR + NHEJ) (lanes 4). The genomic DNA from the set of  $\Delta$ mat1-1-1 transformants was digested with *NheII* and probed with (a) MAT1-1-1 specific, and (b) hygromycin specific probe



**Figure 4.2E.** Southern blot strategy used to determine types of DNA integration in the fungal strains transformed with MAT 1-2-1 KO cassette. The genomic DNA from transformant strains have been digested with *XhoI* and probed with MAT1-2-1 specific probe (black bar) and *hph* gene specific probe (gray bar).



**Figure 4.2F.** Example of types of DNA integrations generate in MAT1-2-1 targeted gene deletion experiments: homologous recombination (HR) (lanes 3, 5, 6, 7, 11, 13, 15, 16, 17, 18 ), ectopic integration (NHEJ) (lanes 4, 12, 14 ), single point recombination (HR + NHEJ) (lanes 8, 9, 10). The genomic DNA from the set of  $\Delta$ mat1-2-1 transformants was digested with *XhoI* and probed with (a) MAT1-2-1 specific, and (b) hygromycin specific probe.

**Table 4.4.** Comparison of SMC and IMC according to the number of transformant per different category of DNA integration and the total number of DNA integration events generated through in each experiment.

Gene	Gene replacement strategy	Number of Transformants/Type of Integration					Total Number of DNA integration events	Total number of transformants analyzed
		HR <sup>1</sup>		NHEJ <sup>2</sup>		HR: NHEJ <sup>3</sup>		
		single	HR+NHEJ	single	multiple			
MAT1	SMC*	10	2	0	8	5	101	25
MAT1	IMC**	5	1	12	5	2	53	25
MAT1	IMC(lp)***	6	0	13	4	2	29	25
MAT1-1-1	SMC	11	6	0	5	4	95	25
MAT1-1-1	IMC	7	4	3	9	2	76	25
MAT1-2-1	SMC	12	1	1	0	6	54	20
MAT1-2-1	IMC	15	3	2	4	0	42	24
MAT1-2-1	IMC(lp)	8	4	3	1	4	38	20

<sup>1</sup>homologous recombination (HR) includes single HR integration events and additional ectopic (NHEJ) integration events of replacement cassette

<sup>2</sup>ectopic integration by non-homologous end joining (NHEJ) event

<sup>3</sup>one flank of replacement cassette is integrated by HR and the other by NHEJ event

\* SMC = split-marker cassette approach

\*\* IMC = intact marker cassette approach

\*\*\* IMC(lp) = intact marker cassette plasmid, linearized in the plasmid sequence

**Table 4.5:** Effect of GC content and the size the gene flanking region and targeted sequences on the efficiency of homologous recombination .

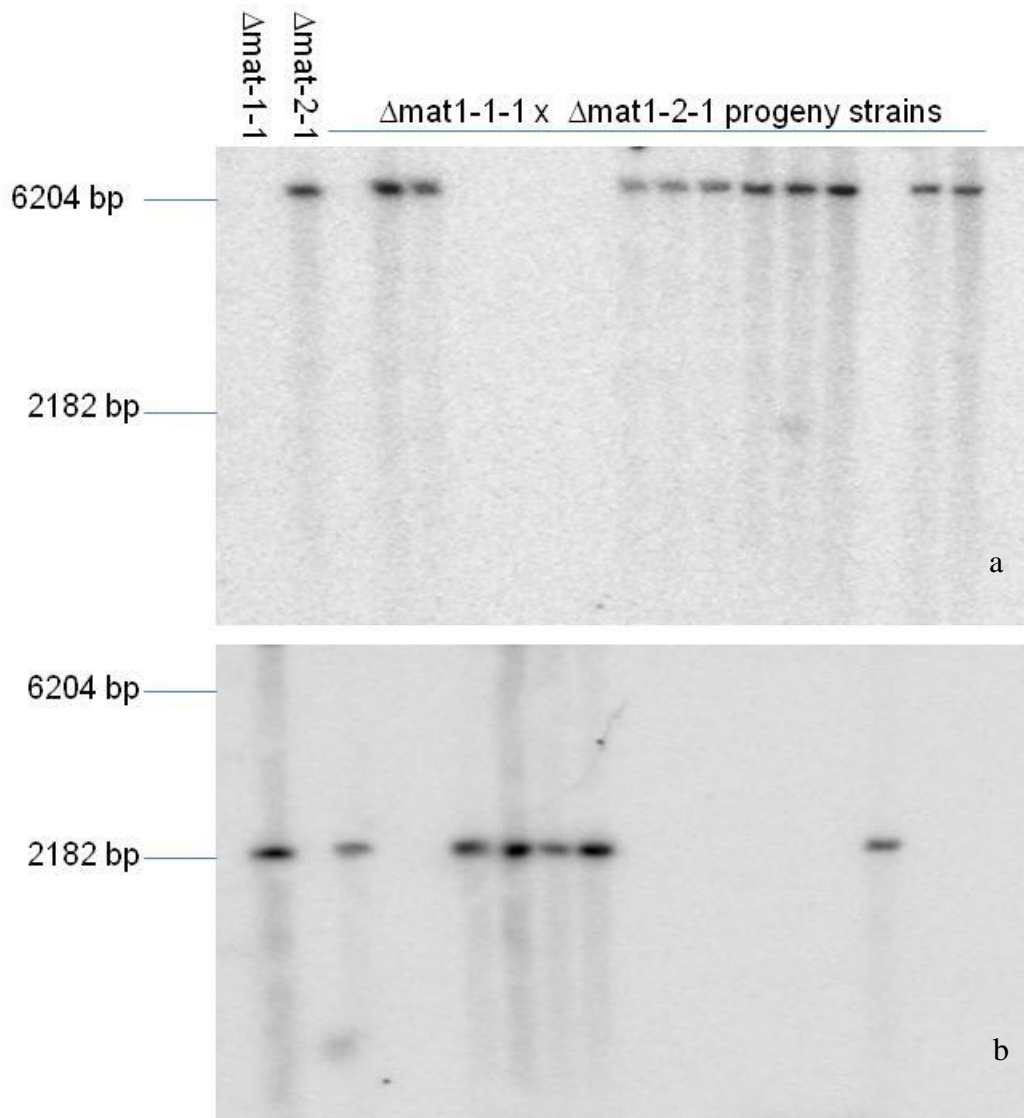
Targeted sequence	GC content (size)			Efficiency of HR <sup>3</sup> *		
	5' flank	gene	3' flank	SMC <sup>1</sup>	IMC <sup>2</sup>	linearized plasmid
MAT1	45.6 % (939 bp)	49.2% (6070 bp)	46.4 % (920 bp)	10/101	15/53	6/29
MAT1-1-1	45.7 % (584 bp)	51.2 % (1117 bp)	48.3 % (582 bp)	11/95	7/76	N/A
MAT1-2-1	45.5 % (898 bp)	52.7 % (978 bp)	47.9 % (1003 bp)	12/54	15/42	8/38

<sup>1</sup>SMC = split marker cassette

<sup>2</sup>IMC = intact marker cassette

<sup>3</sup>HR = homologous recombination

\* Efficiency of HR expressed as the ratio between the number of HR events and the total number of DNA integration events generated by SMC and IMC approach.



MAT1-1-1 : MAT1-2-1 = 6:10;  $\chi^2$  p-value=0.3173

**Figure 4.3.** Segregation of MAT1-1-1 and MAT1-2-1 gene specific markers among the progeny of the  $\Delta\text{mat1-1-1} \times \Delta\text{mat1-2-1}$  cross. a) EcoRI digest of the genomic DNA from  $\Delta\text{mat1-1-1}$ ,  $\Delta\text{mat1-2-1}$  and sixteen heterothallic progeny strains probed with MAT1-2-1 gene specific probe. b) EcoRI digest of the genomic DNA from  $\Delta\text{mat1-1-1}$ ,  $\Delta\text{mat1-2-1}$  and sixteen heterothallic progeny strains probed with MAT1-1-1 gene specific probe. The genomic DNA from the progeny and parental strains were digested with *EcoRI*, probed with MAT1-1-1 specific probe and reprobated with MAT1-2-1 specific probe.

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## CHAPTER 5

### The Role of Mating Type Genes in Pathogenicity of *Gibberella zeae* (*Fusarium graminearum*)

#### 5.1. Introduction

Sexual recombination is a major driving force for the production of genetic diversity across all kingdoms (Zeyl 2009). Sexual development and mating behavior in fungi is under the control of MATing type genes (Casselton 2008). Fungi generally display two different types of sexual behavior: homothallism (self-fertility); and heterothallism (cross fertility) (Bölker 1998; Casselton and Olesnick 1998; Coppin et al. 1997; Fraser and Heitman 2004; Heitman 2006).

Heterothallic species carry different sets of MAT genes that control self / nonself recognition, sexual development, and mating behavior, in separate individuals (B G Turgeon 1998; SH Yun et al. 2000). Successful mating is possible only between individuals with compatible mating types, i.e. "out-crossing". Homothallic species, on the other hand, typically carry different mating type genes regulating sexual development and mating behavior in the same genome. Homothallic organisms preferentially reproduce by selfing, however some have retained the ability to out-cross as well, should the opportunity arise (Coppin et al. 1997). The proportion of out-crossing is usually low relative to selfing (Taylor et al. 1999).

Most filamentous ascomycetes have a single MAT1 locus with two alternative allelic forms, aka. idiomorphs, known as MAT1-1 and MAT1-2 (B G Turgeon and Yoder 2000). The idiomorphs share no DNA homology. MAT1-1 is comprised of three genes, MAT1-1-1, MAT1-1-2, MAT1-1-3. MAT1-2 consists of one or two genes, MAT1-2-1, and MAT1-2-2 (e.g. *Neurospora crassa*) or MAT1-2-3 (e.g. *Gibberella fujikuroi*) (Martin et al. 2011). Each MAT idiomorph contains at least one gene encoding an HMG (high mobility group) DNA-binding motif. In the MAT1-2 idiomorph, the MAT1-2-1 gene encodes the HMG motif, and in the MAT1-1 idiomorph, the HMG-encoding gene is often MAT1-1-3 (Klix et al. 2010). The MAT1-1-1 gene encodes an  $\alpha$ -box DNA



binding motif. Evidence suggests that the products of the MAT1-1-1 and MAT1-2-1 genes interact as heterodimers to produce an active transcription factor that turns on a cascade of genes involved in sexual development (Jacobsen et al. 2002; Metzenberg and Glass 1990; Staben and Yanofsky 1990). Gene deletion studies have shown that the role of MAT genes in sexual development and mating varies somewhat among different species. MAT1-2-1 (HMG) is essential for sexual fertility in all the species in which it has been disrupted, i.e. *Aspergillus fumigatus*, *Neurospora crassa*, *Podospora anserina*, *Sordaria macrospora* and *Gibberella zeae*. MAT1-1-1, which encodes the  $\alpha$ -factor motif, is essential for all afore mentioned species except *Sordaria macrospora*, in which deletion of MAT1-1-2, rather than MAT1-1-1, renders the fungus infertile (Klix et al. 2010).

*Gibberella zeae* (Schwein.) Petch [teleom.], *Fusarium graminearum* [anam.] Schwabe is a homothallic ascomycete, and is a causal agent of the cereal disease known as Fusarium head blight (FHB). FHB results in major economic losses to wheat and barley growers around the world (Goswami, R. S., Kistler 2004; Trail 2009). The FHB epidemic that occurred between 1998-2000 deprived U.S. farmers from the nine major wheat producing states of 2.5 billion dollars in profits (WE Nganje et al. 2002; William Nganje 2003).

Sexual development in *G. zeae* plays an important role in the disease cycle of FHB. The perithecia are produced on overwintering plant debris. The ascospores produced in perithecia are forcibly ejected (Trail et al. 2002), and carried by wind currents or splashed by rain droplets onto flowering wheat heads, hence serving as the primary inoculum for FHB epidemics each season.

Sexual development and mating behavior in *G. zeae* is under control of a single MAT1 locus. The MAT1 locus is comprised of two alternate idiomorphs MAT1-1 and MAT1-2, linked closely together on the chromosome. Lee et al. (2003) demonstrated that, as in *Neurospora crassa* (Ferreira et al. 1998), MAT1-1-1, ( $\alpha$ -factor) and MAT1-2-1 (HMG) are both essential for normal homothallic mating behavior and sexual development. Deletion of either MAT 1-1-2 or MAT1-1-3 reduced fertility, but the mutants were still able to produce ascospores.

The relationship between mating and virulence is well-established among Basidiomycete pathogens, including the human pathogen *Cryptococcus neoformans* and plant pathogens *Ustilago maydis* and *Ustilago hordei*, and in the Ascomycete pathogen *Candida albicans*.

*Cryptococcus neoformans* has two mating types, known as mat a and mat  $\alpha$ . The mat  $\alpha$  mating type is found 30-40 times more frequently in nature in comparison with mat a (Kwon-Chung 1992). The mat  $\alpha$  mating type is also much more virulent than mat a in a mouse model. Comparative studies of mat a and mat  $\alpha$  in isogenic, well-characterized *C. neoformans* strains show that additional genes contained within the mat  $\alpha$  idiomorph contribute to the differences in virulence between strains (Niesen et al, 2005).

In *Ustilago maydis* two loci, a and b, are in control of mating. Mating of haploid strains differing in both a and b results in formation of a filamentous dikaryon that can invade and induce tumor formation in the maize host. The pheromone response factor coordinates filamentous growth and pathogenicity in *U. maydis* (Hartmann, Kahmann and Bolker, 1996). Targets of the MAT gene products, known as fuz1, fuz2 and rtf1, are necessary for the filamentous transition and tumor formation (Bannuet, 1991).

*Candida albicans* is one of best examples of a role for MAT genes in fitness and pathogenicity of an Ascomycete pathogen. *C. albicans* switches between two colony phenotypes, known as white and opaque. The white phase results after mating, controlled by the MAT locus. Mating is promoted under stressful conditions (i.e. elevated CO<sub>2</sub> levels) (Huang et al, 2009). Heterozygous individuals (a/ $\alpha$  genotypes) resulting from mating are significantly more virulent than either a/a or  $\alpha$ / $\alpha$  homozygous individuals (Lockheart et al, 2005, Miller et al, 2002).

There are indirect lines of evidence that suggest the possibility that MAT loci also play roles in pathogenicity of plant-pathogenic ascomycetes. In the cosmopolitan wheat pathogen *Mycosphaerella graminicola*, MAT1-1 strains are 14–22% more pathogenic than MAT1-2 strains in greenhouse experiments (Jiasui Zhan, Torriani, et al. 2007). In 2002 the same research group found that MAT1-2 and MAT1-1 mating types were generally equally distributed across different geographic regions and spatial levels (J Zhan et al. 2002), but they noted that the frequency was skewed towards MAT1-2 in two fields that had been treated with a fungicide close to the sampling date. Based on that

observation, they hypothesized that the MAT1-2 mating type might be associated with fungicide tolerance. In another study, 13% of *M. graminicola* strains recovered from a resistant cultivar in an inoculated field experiment were sexual recombinants, compared with only 9% of the strains isolated from a susceptible cultivar, suggesting that sexual recombination facilitated pathogen adaptation to host resistance factors (J Zhan, Mundt, et al. 2007).

Many plant-pathogenic fungi that are phylogenetically affiliated with the Ascomycetes do not seem to reproduce sexually, yet they still carry highly-conserved and fully functional MATing type genes (Alvarez-Perez et al. 2010; Arie et al. 2000; Stefanie Pöggeler 2002; Sharon et al. 1996; B G Turgeon et al. 1995; Varga 2003; SH Yun et al. 2000). This may be due to the presence in these species of cryptic sex, as is most often suggested, but alternatively it could suggest the possibility that MATing type genes have an important role besides the one in mating in these pathogenic fungi.

The first hint that MAT1 genes play an important role in pathogenicity of *G. zeae* came from a study published by Desjardins et al. (2004). These authors found that deletion of the whole MAT1 locus had no significant effect on aggressiveness in experiments on spring wheat in the greenhouse, however the MAT1 deletion mutants were unable to initiate FHB epidemics in the field. The explanation was that this was due to the absence of ascospores that could serve as primary inoculum. However, the deletion strains still produced plenty of macroconidia, which can also serve as inoculum. So another possibility was that MAT genes play an important role in providing a competitive advantage under field conditions.

In their cytological study of the development and differentiation of *G. zeae* during colonization of wheat stems, Guenther and Trail (2005) observed thin, uninucleate hyphae together with much thicker hyphae that appeared to be dikaryotic. The thicker hyphae were associated with colonizing behavior as they traversed the wheat stem and rachis lumen, facilitating rapid movement of the pathogen through the host tissues. The dikaryotic hyphae were also found associated with production of perithecia, and thus could be under the control of MAT genes.

In a microarray study, 59% of expressed genes were significantly down-regulated in a *mat1-2-1* deletion mutant strain of *G. zeae*, compared with the WT strain (Lee et al., 2006). These included genes involved in stress response, metabolism, and development, all of which could potentially be significant in pathogenicity. Protein expression profiles of *mat1-2-1* deletion strains showed a similar trend, with 11 proteins down-regulated and two up-regulated in the deletion strains. Six of the differentially expressed proteins were related to cell-wall structural proteins, and the rest were metabolic and environmental stress response-related proteins (S-H Lee et al. 2008). Again, changes in any of these processes could alter pathogenicity.

The hypothesis that is the basis for the work described in this chapter is that the MAT genes of *G. zeae*, as master regulators, also regulate genes that are important for pathogenicity. The objective was to delete the MAT genes in the genome-sequenced strain PH-1 and study the effect of the deletions on aggressiveness of *G. zeae* on a susceptible SRWW variety, Pioneer 2555.

## **5.2. Materials and Methods**

**Fungal strains and growth conditions:** All fungal strains were routinely grown at 23° C with constant light (Sylvania F032/741/ECO) . Mutant strains were single-spored and stored on silica gel at -20° C or -80° C (Tuite, 1969, after Perkins, 1962). Strains were never subcultured more than once. Spore production was evaluated on two different types of media. Production of asexual spores (macroconidia) was assessed after 7 days of growth on mungbean agar [40 g mungbeans and 10 g Bacto® Agar per L (Bai and Shaner 1996)]. Production of sexual spores (ascospores) was measured after 21 days of growth on carrot agar media [400 g carrots, 10 g Bacto Agar per L (Klittich and Leslie 1988)]. In each case, 2 ml of sterile water was applied to the surface of the Petri plate and the spores were released by rubbing with a sterile plastic micro-pestle. The spore suspension was filtered through glass wool to remove mycelial fragments. Spores were counted by using a hemocytometer. For use as inoculum, spores were washed once in sterile water and the concentration was adjusted as necessary.

**Generation of MAT deletion (knock-out) strains:** All gene deletion (knock-out, KO) mutants and ectopic strains were generated in the *Gibberella zeae* strain PH-1 (NRRL 31084) wild-type background. The MAT1-1-1 and MAT1-2-1 genes, and the complete MAT 1 locus, were deleted by using either a split-marker or an intact marker gene targeting strategy (described in Chapter 4 of this Dissertation).

**DNA extraction:** To isolate fungal DNA, five ml of YEPD media (20 g dextrose, 10 g Bacto® peptone, 3 g yeast extract per L) in 50 ml glass tubes was inoculated with an 8-mm plug of an actively growing culture and incubated for 5-7 days at room temperature with agitation (250 rpm). The mycelial mats were harvested by filtration, frozen at -80°C for one hour, and lyophilized in a freeze-drier. Lyophilized tissue was pulverized with steel beads in 96-well plates in a GenoGrinder® 2000 (500 strokes/s) for 1 minute. Pulverized tissue was suspended in lysing buffer (5 M NaCl, 1M Tris-HCl, pH7.5; 0.5M EDTA, 10% SDS) for 30 minutes at 65°C. The solution was subsequently extracted with 1 volume of phenol:chloroform:isoamyl alcohol (25:24:1) for an additional 30 min at 65°C. The resulting slurry was centrifuged for 20 min at 13,000 rpm to separate the phases. The aqueous phase (500-600 µl) was transferred into a clean Eppendorf tube and precipitated with 0.6 volume of isopropanol. The samples were centrifuged for 10 min at 13,000 rpm to pellet the DNA. The pellet was washed twice with 70% ethanol. After the ethanol washes, the DNA pellet was dried for 10 minutes in a transfer hood, then dissolved in 100 µl TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0 ) + 10 µg RNaseA at room temperature.

**Southern hybridization analysis:** The Southern blot analysis was performed by using standard molecular biology protocols. Briefly, 500 ng of DNA from each strain was digested with selected restriction enzyme; *NheI* was used for MAT1-1-1 KO transformants, and *XhoI* was used for MAT1-2-1 and MAT1 KO strains. The digested DNA fragments were separated on a 0.8 % agarose gel in 0.5 x TBA buffer. Separated

fragments were transferred onto positively charged nylon membrane using an electroblotting device "Genie electroblotter" (Idea Scientific, Minneapolis, MN ). The DNA fragments were denatured by treating the nylon membrane with 0.4 N NaOH, and cross-linked with UV light. After prehybridization the membranes were probed with a set of gene-specific probes, and after the data were collected the membranes were stripped and re-probed with a hygromycin-specific probe. The Southern blot scheme for each of the targeted genes presented in Figure 5.1, 5.2, and 5.3.

**Crossing procedure and marker segregation analysis:** MAT KO strains were crossed by using the mycelial plug method described by Bowden and Leslie (1999). Five-mm plugs of mycelia of each parental strain, taken from the edges of actively growing cultures on potato dextrose agar (PDA), were placed on opposite halves of a 60 mm Petri plate. After four days of incubation at 23° C, perithecial production was induced by applying 500-1000 µl of 2.5% Tween 60 to the surface of each plate, and gently rubbing the aerial mycelium with a sterile glass rod to flatten it. Following induction, the plates were incubated at 23° C with constant fluorescent light until perithecial maturation. The mature perithecia extruded cirrhi that contained several thousand ascospores 2-3 weeks after induction. A sterile glass needle was used to pick up individual cirrhi, which were then dispersed in water and plated on 2% water agar. After 10-14 hours of growth on water agar, isolates arising from single ascospores were transferred to individual 6 mm Petri plates.

**Molecular probes and segregation analysis in the progeny of a  $\Delta$ mat1-1-1 x  $\Delta$ mat1-2-1 cross:** A set of twenty single-spored progeny strains was derived from five separate perithecia from a cross of  $\Delta$ mat1-1-1 x  $\Delta$ mat1-2-1 KO strains. Isolated DNA was restricted with *BsoBI* and probed with the repetitive RFLP probe REP1.92. The same set of DNAs was restricted with *XhoI* and probed with MAT1-1-1- and MAT1-2-1-specific probes to analyze segregation of mating type genes. Segregation ratios among the two mating type genes and the various RFLP markers within the population of progeny strains were statistically evaluated by using a  $\chi^2$ -test.

**FHB pathogenicity assay:** The susceptible soft red winter wheat variety Pioneer 2555 was used for this study. Wheat seeds were planted in a mixture of topsoil (Maury silt loam) and PromixBX growth substrate (3:2) in a 72-cell format at the rate of three seeds per cell. The seeds were lightly covered with a moist layer of soil mixture. Seeds were germinated in the greenhouse for 3-5 days at ambient temperature of 25 °C, and a 12 h photoperiod. Shortly after germination and seedling emergence, seedlings were treated with a systemic fungicide NOVA<sup>TM</sup> 40W (Dow AgroSciences Canada Inc.) at recommended rate against powdery mildew, and transferred to a cold room (~4 °C) with constant fluorescent light for 8 weeks. At the end of the vernalization period, the seedlings were transplanted in cone containers filled with a mixture of topsoil (Maury silt loam) and PromixBX (3:2). Transplanted wheat plants were grown in greenhouse with a 14/10 photoperiod, provided by "Hortilux" LU430S/HTL/EN high pressure sodium lights, and ambient temperatures between 25-28°C. Transplants were fertilized with 150 ppm of N:P:K (20:10:20) fertilizer formulation every 14 days starting two weeks after transplanting with last fertilization at heading. Flowering typically occurred after 3-4 weeks. At early- to mid- anthesis, a single centrally positioned floret on the primary flowering stem of each plant was inoculated with 10 µl of a spore suspension as described by (Miedaner et al. 2003). Symptom severity was recorded at seven and ten days post-inoculation, as the number of FHB symptomatic spikelets per inoculated spike.

**DON production in *planta*:** Inoculated plants were grown in the greenhouse to physiological maturity, then whole heads were harvested, threshed and cleaned by hand, and the seeds sent for GC-MS mycotoxin analysis. The analysis was performed by Dr. Yanhong Dong at the USDA ARS Cereal Disease Lab, St. Paul, MN (Fuentes et al, 2005; Mirocha et al, 1998).

**DON production in *media*:** For estimation of deoxynivalenol production in liquid media a modified protocol of Miller et al, 1983, was used. Briefly, 5 ml of 2% GYEP (2% D-glucose, 0.1% yeast extract, 0.1% peptone) was inoculated with 1 ml of spore suspension at the concentration of  $5 \times 10^4$  spores/ml. The liquid cultures were incubated for 14 days

in dark at the ambient temperature with constant agitation (200 rpm). After the 14-day incubation period, 1 ml of growing liquid culture was filtered through a glass wool filter. A 500- $\mu$ L subsample was freeze-dried and sent for GC-MS mycotoxin analysis by Dr. Yanhong Dong at the USDA ARS Cereal Disease Lab, St. Paul, MN (Fuentes et al, 2005; Mirocha et al, 1998).



### 5.3. Results

To address the hypothesis that MAT genes play a role in pathogenicity of *G. zeae*, three sets of gene deletion mutant strains and ectopic control strains were produced. These deletions encompassed the entire *MAT1* locus of *G. zeae*, the *MAT1-1-1* gene alone, or the *MAT1-2-1* gene alone. The genetic identity of the mutant and ectopic strains was confirmed by Southern hybridization analysis (Figures 5.1-5.3). For each of the targeted loci, two independent KO strains and two ectopic control strains were selected for analysis.

**Development *in vitro*:** Deletion of any of the three MAT loci resulted in mutant strains unable to reproduce by homothallic "selfing". The gene deletion mutant strains were all capable of producing perithecial initials. There were no noticeable differences between the MAT mutant strains, ectopic controls and WT strains in the number of perithecial initials produced. When crushed, the initials produced by mutant deletion strains contained no asci or ascospores. All of the ectopic strains retained the WT fertility phenotype: after induction on carrot agar media all of them produced fertile perithecia containing asci with eight ascospores per ascus (Figure 5.4). The diameter of the perithecial initials produced by the mutants was statistically reduced in comparison to the perithecia developed by the WT and ectopic strains (Figure 5.5).

In culture, the WT PH-1 strain of *G. zeae* produced hyphae with two distinct morphologies: thin, mononucleate; and thick, multinucleate (often binucleate). The thick hyphae frequently produced coiled structures that eventually gave rise to perithecial initials. There were no noticeable differences between mutant KO, ectopic controls, and WT strains in the production of thin and thick hyphae, or in the production of the coiled structures (Figure 5.6). There were no statistical differences in the numbers of mononucleate, multinucleate or binucleate cells produced by any of the strains (Figure 5.7).

There was a significant increase in macroconidial production among all the mat deletion strains grown on carrot agar, in comparison with the WT and ectopic strains (Figure 5.8).

The ectopic strains did not differ from the WT in the production of either ascospores or conidia (Figure 5.8).

The heterothallic sexual behaviour of the mutant strains was tested by crossing them in various combinations and analyzing segregation ratios of molecular markers among the progeny of the crosses. The MAT loci segregated 1:1 among progeny of a cross of the *mat1-1-1* KO (in the PH-1 background) and a *MAT1-2-1* KO (also in the PH-1 background) (see figure 4.3 in this dissertation). A cross between another *mat1-1-1*KO (generated in a Gz3639 background, see Appendix 1 of this dissertation) and *mat1-2-1*KO (generated in the PH-1 background) strains also resulted in production of fertile perithecia containing asci with eight ascospores per ascus (Figure 5.9). CAPs markers *PstI2* and *EcoRI3* segregated in a 1:1 ratio, as expected (Figure 5.10). When each of the KO mutants was crossed with the *mat1*KO strain, only perithecial initials were produced, and no fertile perithecia developed (not shown).

Growth in culture of all of the strains except for one of the ectopics appeared normal. However, one ectopic strain (*MAT111E2*) frequently produced fast growing aconidial sectors with abundant aerial mycelium. Due to this apparent instability, which led to some difficulties in obtaining consistent numbers of macroconidia over time, this ectopic strain was not included in the pathogenicity and toxigenicity experiments, described below.

**Pathogenicity:** In a single large greenhouse experiment that included fifteen replications per treatment, the deletion of the complete *MAT1* locus had no significant effect on aggressiveness to the susceptible winter wheat variety Pioneer 2555 (Figures 5.11, 5.12). In pair-wise comparisons, the only statistically significant difference (other than with the water control) was between the WT strain PH-1 and one of the ectopic control strains (Table 5.1). Deletion of *MAT1-1-1* alone resulted in a significant reduction in aggressiveness in comparison with the WT strain PH-1 or the ectopic strain (Figure 5.13, 5.14, Table 5.2). The *mat1-1-1* KO strains were not significantly different from the water controls (Figure 5.13, 5.14, Table 5.2). Deletion of *MAT1-2-1* alone resulted in a somewhat less dramatic, but still significant, reduction in aggressiveness in comparison

with PH-1 (Figure 5.15, 5.16, Table 5.3). One of the ectopic control strains was not significantly different from one of the KO strains. The second ectopic strain was equivalent to the WT and significantly different from both mutants.

**Toxin production:** The deletion of the whole MAT1 locus had no effect on DON production *in planta* (Figure 5.17, Table 5.4), or in media (Figure 5.18, Table 5.5). In contrast, the deletion of the MAT1-1-1 gene alone had a significant negative effect on DON production *in planta* (Figure 5.19, Table 5.6), when compared with the WT PH-1 and the ectopic control strain. The MAT1-1-1 deletion mutants were also significantly reduced in their ability to produce DON in media when compared with the WT PH-1 (Figure 5.20, Table 5.7). However, neither KO mutant was statistically different from the ectopic strain, so I cannot conclude that the MAT1-1-1 deletion is responsible for this reduction.

The effect of a MAT1-2-1 deletion on DON production *in planta* was inconsistent (Figure 5.21, Table 5.8). One of the KO strains was significantly reduced in its ability to produce DON, but the other was statistically equivalent to the WT. Furthermore, both ectopic strains were significantly different from the WT, with one producing more and the other less DON *in planta* (Figure 5.21, Table 5.8). In media, one of the KO strains was equivalent to the media control and produced statistically less DON than the WT. However, this strain was not statistically different from the ectopic controls, and none of the other pair-wise comparisons revealed a statistical difference. (Figure 5.22, Table 5.9).

## 5.4. Discussion

Sexual recombination is one of the main ways in which genetic variability is generated in a species. MATing type genes regulate sexual behavior in fungi. As master transcriptional regulators, mating type genes impact the expression of many other genes, hence directly or indirectly affecting important life processes including metabolism, stress responses, and differentiation. The current study focused on exploring the potential role of MAT genes in pathogenicity-related phenotypes of *G. zeae*. There are several examples for a direct role of MAT genes in pathogenicity among Basidiomycetes, including the human pathogen *Cryptococcus neoformans* (Idnurm, Reedy, Nussbaum, and Heitman, 2004; Kwon-Chung, Edman, and Wickes, 1992; Okagaki and Nielsen, 2009; Perfect, Wong, Chang, Kwon-Chung, and Williamson, 1998) and the plant pathogen *Ustilago maydis* (Bakkeren, Kämper, and Schirawski, 2008; Banuett, 1991; Brefort et al., 2009; Feldbrügge, Kämper, Steinberg, and Kahmann, 2004; Moore and Edman, 1993), where MATing type genes are known to act directly as virulence factors. In Ascomycetes, indirect evidence of a role for mating type genes in virulence includes differences in aggressiveness between mating types in the plant pathogen *Mycosphaerella graminicola* (Zhan, Kema, Waalwijk, and McDonald, 2002; Zhan, Torriani, and McDonald, 2007); and the fact that fertile (opaque) strains of the human pathogen *Candida albicans* are more pathogenic than non-fertile (white) strains (Bennett, 2009; Lockhart et al., 2005; Miller and Johnson, 2002).

Desjardins et al, (2005) deleted the entire MAT1 locus of the Gz3639 strain of *G. zeae*. They found that the mutants did not produce perithecia, and could not mate with fertile strains. They also reported that the mutants were not consistently affected in either pathogenicity or DON production in greenhouse assays on spring wheat. However, when the MAT1 KO mutants were tested in the field, they were unable to cause a FHB epidemic (Bai, Desjardins, and Plattner, 2002; Desjardins et al., 2004). It was suggested that this was due to the lack of forcibly discharged ascospores that could serve as the primary inoculum. In another study, Lee et al. (2002) deleted the MAT1-1-1 and the MAT1-2-1 genes individually, also in the Gz3639 background. These deletions also resulted in self-sterility, but unlike the MAT1 whole-locus KO mutants, these mutants

were able to cross with each other, and with their WT progenitor. They went on to analyze changes in expression of genes and proteins in the MAT1-2-1 KO strain ( Lee et al., 2008; Lee et al., 2006) Genes involved in metabolism, stress response, and cell wall development and differentiation were all down-regulated in the MAT1-2-1 KO strain during sexual development. Since many of these genes could play significant roles during the host-pathogen interaction, it seemed possible that the MAT1-2-1 KO could be altered in pathogenicity. However, Lee et al. did not do pathogenicity assays with the mutants.

The mutant and complemented strains produced by Desjardins et al. are unfortunately no longer available. I also was unable to obtain the mutants reported by Lee et al., although I did receive two other strains, only one of which proved to be an actual MAT KO in the Gz3639 background (Appendix 1 of this Dissertation). To test the hypothesis that the MAT genes play a role in fungal pathogenicity I produced a new set of mutant deletion strains and appropriate control strains in the *G. zeae* strain PH-1, which has a well-characterized genetic background, and a high-quality public genome sequence.

As expected, all of the PH-1 MAT KO mutants were self-sterile, but capable of producing perithecial initials. This confirms that the MAT genes do not control the ability of *G. zeae* to initiate development related to fertility, but only to produce asci and ascospores. The deletion of any of the MAT loci enhanced the asexual fecundity in mutant strains, which produced significantly higher numbers of macroconidia in comparison to WT and ectopic strains. This was not reported in previous studies of *G. zeae* MAT KOs, and so it may be a feature of the PH-1 strain that is not true for Gz3639. In *A. nidulans*, where the relationship between sexual and asexual development has been studied most thoroughly, it appears that there is a signaling switch that controls production of sexual versus asexual spores (Kim et al., 2002). Production of secondary metabolites is also linked to that switch, and correlated with asexual sporulation (Calvo et al., 2002). This could provide one explanation for the prevalence of asexual strains among plant pathogens: in the absence of some advantage of sex, increased fitness of asexual strains may cause the population to shift from predominantly sexual to predominantly asexual. Pathogens like *G. zeae* are exceptional in maintaining sexual

reproduction as a major part of their lifecycle: for this fungus the advantages of sexuality must outweigh any possible disadvantages of lower asexual fecundity.

The MAT1 KO mutants were not able to self, nor could they mate with either of the idiomorph KO strains, thus they were completely sterile. The MAT1-1-1 and MAT1-2-1 KO strains, in contrast, were fertile but obligately heterothallic, as previously reported by Lee et al, (2003). Thus, besides being useful for studying the role for deleted genes on pathogenicity, these strains are valuable tools for genetic studies because they substantially increase the frequency of sexual recombination. I tested if there was a difference between crossing mutants produced in the same genetic background versus mutants from different backgrounds by comparing a cross of the two PH-1 mutants with a cross involving the PH-1 mat1-2-1 KO strain and the mat1-1-1KO created in Gz3639. Segregation of molecular markers was 1:1 in both crosses. There were no obvious differences in the numbers of perithecia produced, or the numbers of normal eight-spored asci produced. Thus there was no evidence for “inbreeding depression” that sometimes results after sib-crossing or back-crossing heterothallic organisms for one or more generations (Uyenoyama, M.K., 1988a,b). Inbreeding depression is thought to result from loss of heterozygosity at loci other than the MAT genes that affect fertility: perhaps in the homothallic *G. zeae*, these types of loci are not common.

I evaluated the aggressiveness of the mutant and control PH-1 strains on the susceptible SRWW variety Pioneer 2555. There has been no previous published record of the effect of individual idiomorph deletions in *G. zeae* on FHB severity. The deletion of the complete MAT1 locus had no effect on the level of aggressiveness, similar to the result reported by Desjardins et al. for their MAT1 KO of Gz3639 (Desjardins et al., 2004). In contrast, deletion of either MAT1-1-1 or MAT1-2-1 resulted in a significant reduction in aggressiveness. The effect was particularly dramatic for the MAT1-1-1 KO mutants, for which the level of disease produced was not statistically different from the water controls. The results presented in this study are limited by the fact that they represent only one experiment. Unfortunately, due to some difficulties producing a healthy winter wheat crop in the greenhouse, and presence of insect pests, I was unable to replicate the greenhouse experiment. Nonetheless, the results of the single experiment are strongly

suggestive since each treatment was repeated 15 times within the experiment, and two independent mutants were tested along with all the necessary control strains.

In heterothallic ascomycetes, the MAT1-1-1 and MAT1-2-1 genes form heterodimers that activate genes involved in recognition and subsequent sexual development (Coppin et al., 1997). In homothallic ascomycetes, both genes are usually present in a single individual and the evidence suggests that their products also interact to produce heterodimers to activate sexual development (Jacobsen et al, 2002). My findings, and those of the previous studies, confirm that both genes must be present for sexual development in *G. zeae* to occur. Interestingly, my findings also suggest that the presence of either gene product alone, in the absence of the other, has a negative effect on pathogenicity. Deletion of all the MAT genes together does not have a similar effect, thus suggesting that it is the activity of each gene alone, and not the absence of the heterodimer, that is important. The effect is especially pronounced when only the MAT1-2-1 gene product is present in the MAT1-1-1 KO. There is some evidence that a single MAT protein alone can produce a homodimer that may have regulatory activity (Jacobsen et al., 2002). Thus it could be the activity of this homodimer that causes the negative effect on pathogenicity. A negative effect of the MAT homodimers on pathogenicity could explain the importance of homothallism in the life cycle of this organism in relation to disease and survival.

In their histological and cytological study of the infection process of *G. zeae* on wheat, Guenther et al, (2005) described two main hyphal morphologies: thin, mononucleate hyphae; and thick multinucleate hyphae that were associated with production of perithecia. Guenther et al. reported that the multinucleate hyphae were mainly dikaryotic, suggesting the possibility that they were generated through conjugate division, normally controlled by the MAT genes. The presence of an extended dikaryon would be highly unusual for an Ascomycete, although it is the norm for Basidiomycetes. I tested the hypothesis that the deleted MAT genes have an effect on production of similar multinucleate hyphae produced on carrot agar, an inductive medium for sexual development. However I saw no difference between mutants, WT and ectopic control strains in their ability to produce thick or thin hyphae, or the coiled structures originating from the thick hyphae that preceded development of perithecial initials. The thick hyphae

did contain cells with two nuclei, but they did not appear to be a true dikaryon since many of the cells contained fewer or more than two nuclei. It is still possible that a difference would be found *in planta*: future work should examine this question. In more recent articles, Guenther and Trail have suggested that the thick multinucleate hyphae, which are rich in lipid bodies, function as overwintering and support hyphae for perithecial development (Guenther et al., 2009). It seems that if these hyphae do have a role in sexual development, it relates to vegetative aspects of the production of the perithecial initials, rather than to the production of ascogenous hyphae, asci, and ascospores. The question of whether ascogenous (dikaryotic) hyphae associated with the hymenial layer of the perithecia are affected in the mutants was not addressed in my study.

Deoxynivalenol is the best-known and most well-researched aggressiveness factor in *G. zeae*. I tested the effect of the MAT gene deletions on DON production *in planta* and *in vitro*. Deletion of the individual idiomorphs (but not the entire MAT locus) reduced the accumulation of DON *in planta*. It is not clear if the effect on DON production was direct, although I note that the MAT1-1-1 mutants also produced very low amounts of toxin *in vitro*, thus suggesting that presence of the MAT1-2-1 gene product alone somehow negatively influences that production, perhaps through effects on gene expression.

More work is needed to follow up on all of these intriguing experiments. The results of the pathogenicity assays need to be confirmed in wheat and in corn stalks, where preliminary studies suggested that the MAT1-1-1 KO strains were also less aggressive. The strains that I produced in this chapter will be useful for future analysis of the role of MAT genes individually and together in mating and in regulation of genes that may be important for pathogenicity. Because they are all made in the same background, and in a strain that has a high quality genome sequence, they will be very valuable for this. This is especially true given that other published strains are unavailable. Indeed, my strains have already been shared with other members of the community for studies on the role of the MAT genes in development.



## 5.5. References

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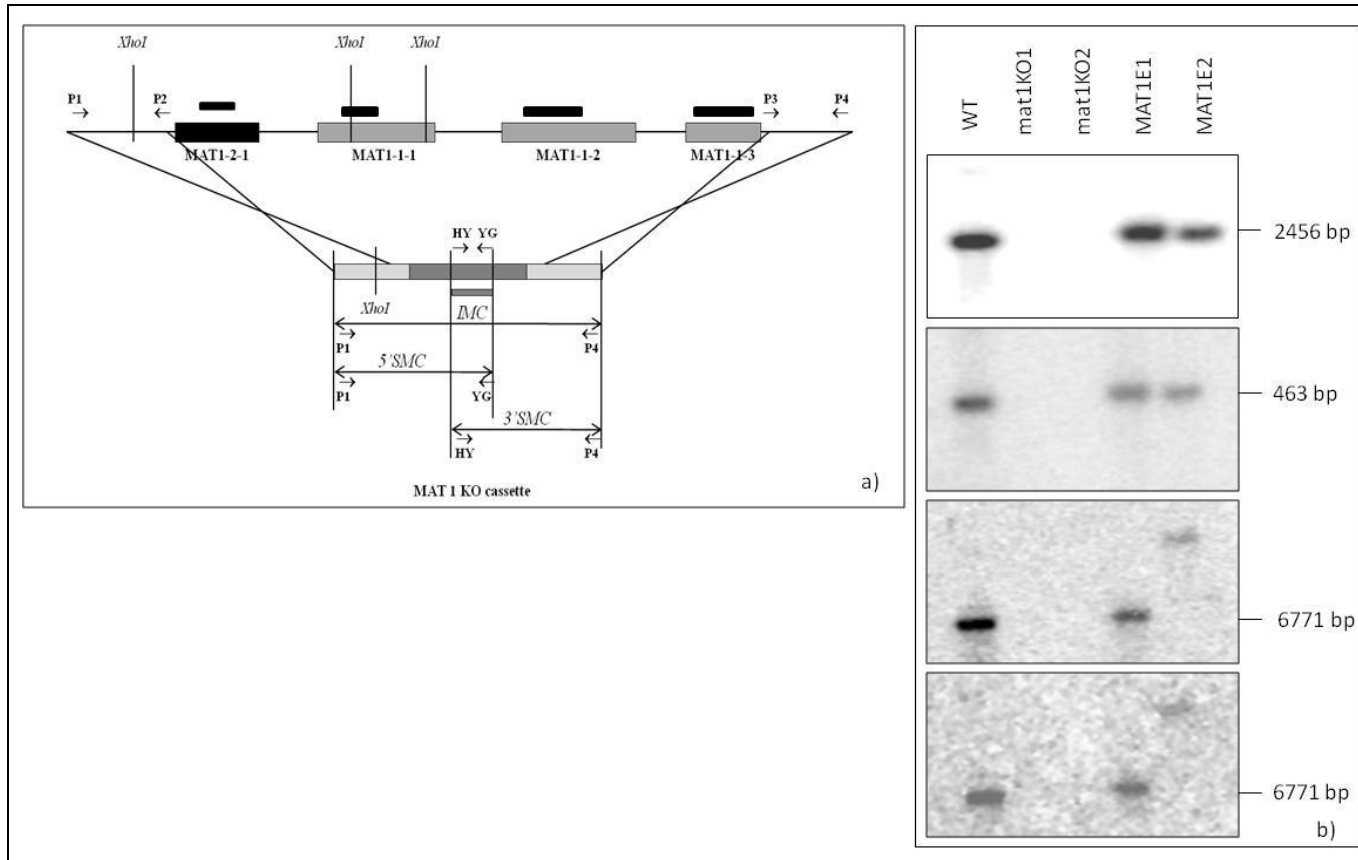
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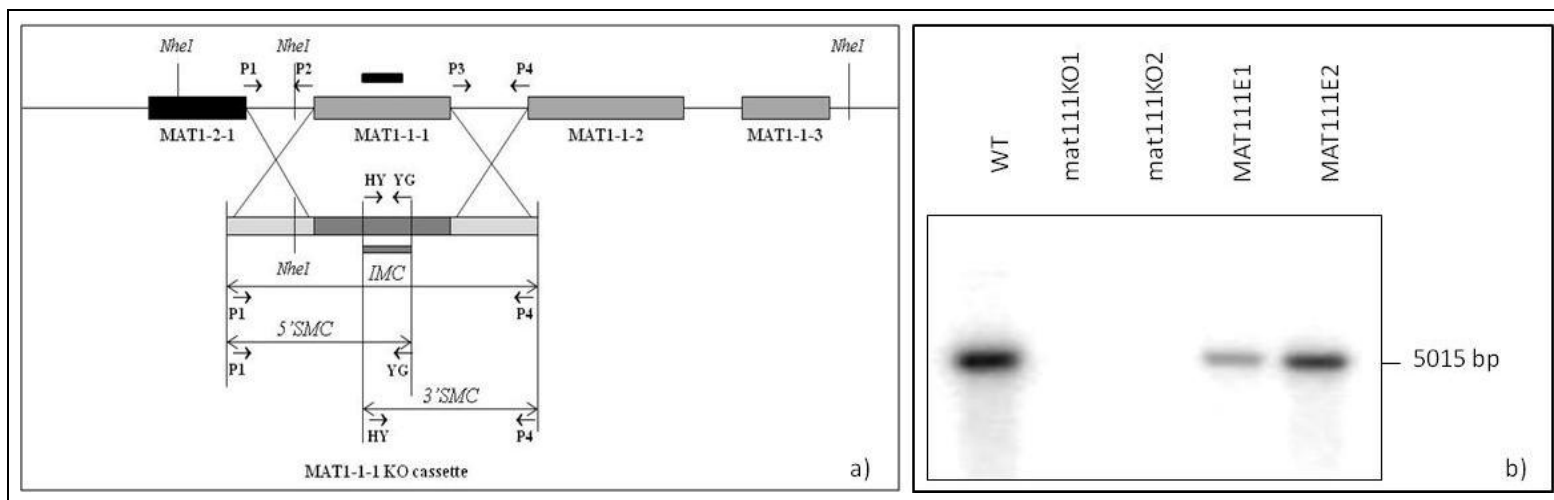
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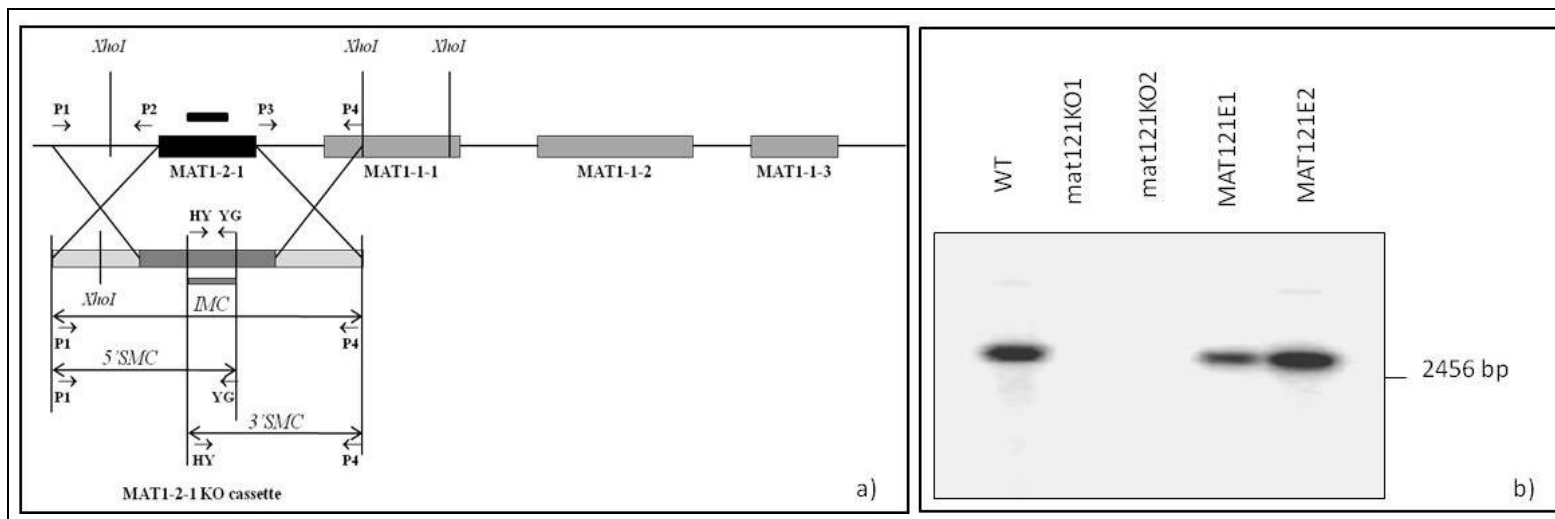


**Figure 5.1:** The Southern blot strategy used to confirm the identity of MAT1 KO mutants and ectopic strains. a) Genomic DNA was digested with *XhoI* and probed with a gene-specific probe (black bar). b) Southern hybridization results after probing with gene-specific probes (from top to the bottom): MAT1-2-1, MAT1-1-1-, MAT1-1-2, and MAT1-1-3.

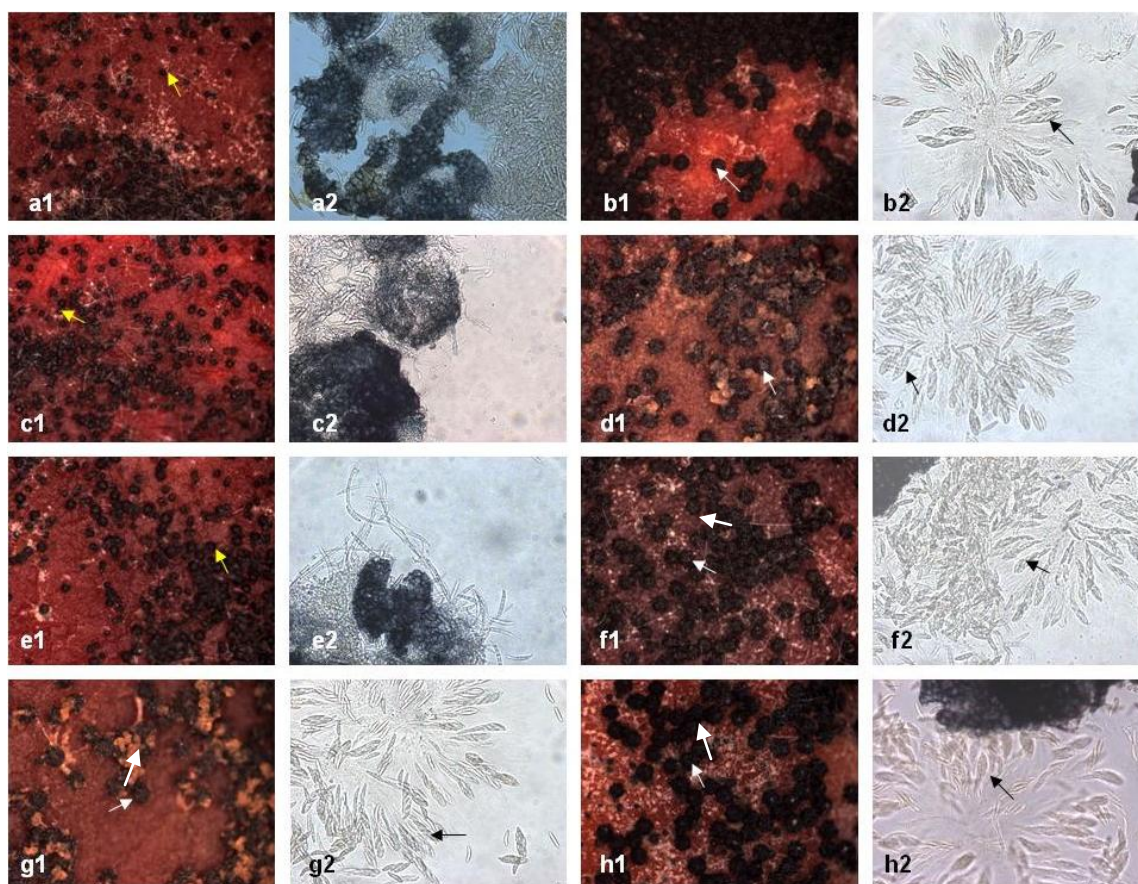


**Figure 5.2:** The Southern blot strategy used to confirm the identity of MAT 1-1-1 KO mutants and ectopic strains. A) Genomic DNA was digested with *NheI* and probed with a MAT1-1-1 gene-specific probe (black bar). B) Southern hybridization results after probing with a MAT1-1-1 gene-specific probe.

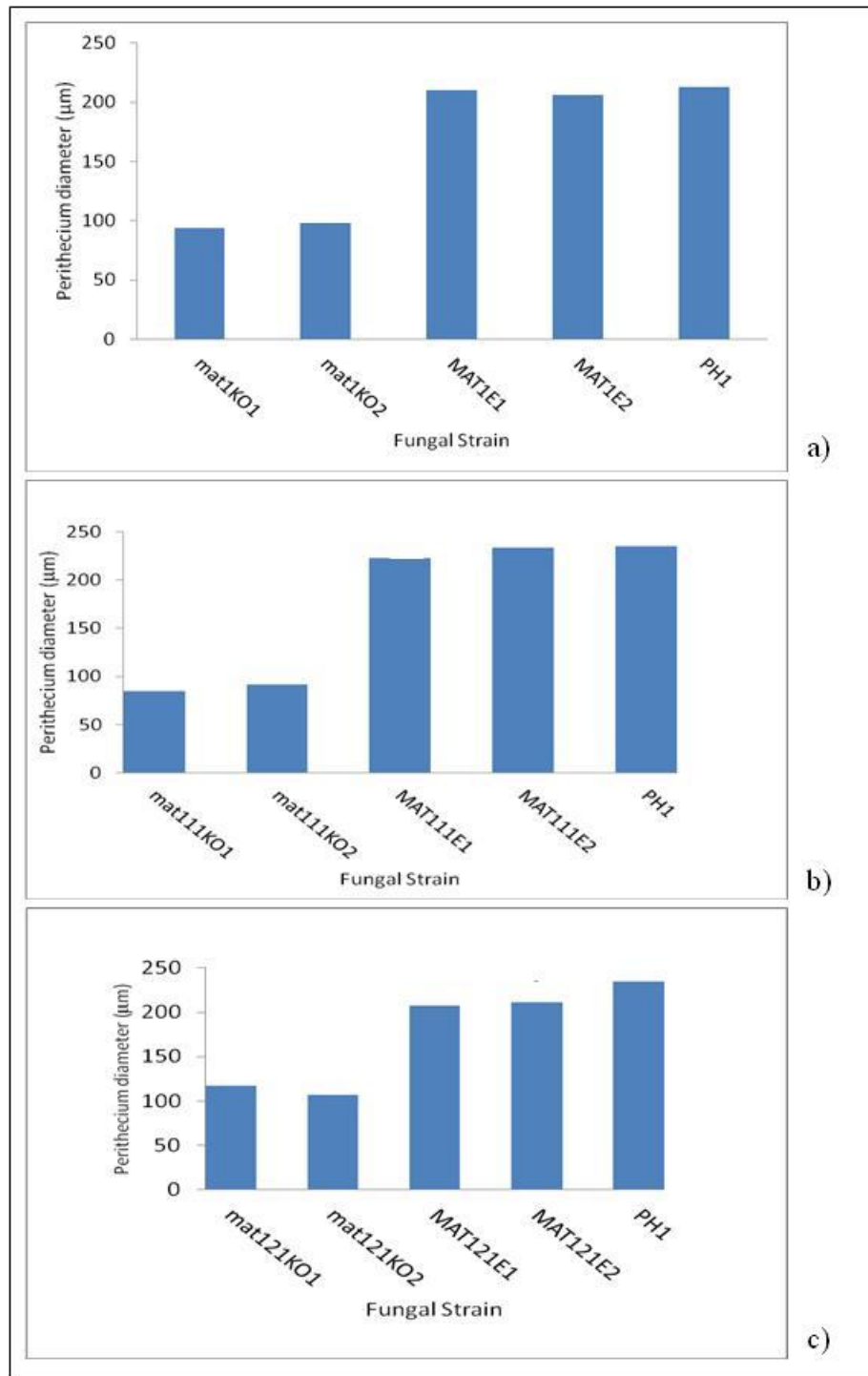




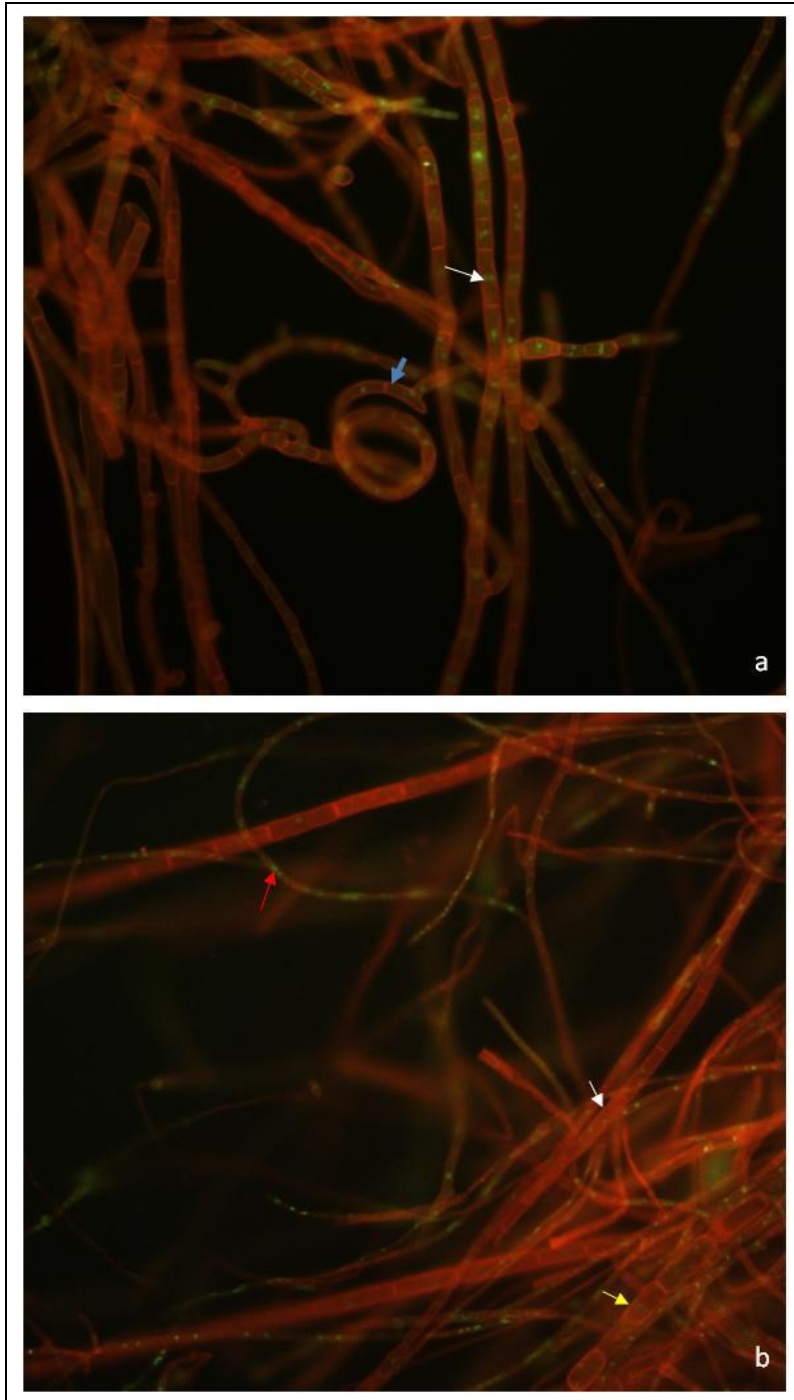
**Figure 5.3:** The Southern blot strategy used to confirm the identity of MAT 1-2-1 KO mutants and ectopic strains. A) Genomic DNA was digested with *XhoI* and probed with a MAT1-2-1 gene specific probe (black bar). B) Southern hybridization results after probing with a MAT1-2-1 gene-specific probe.



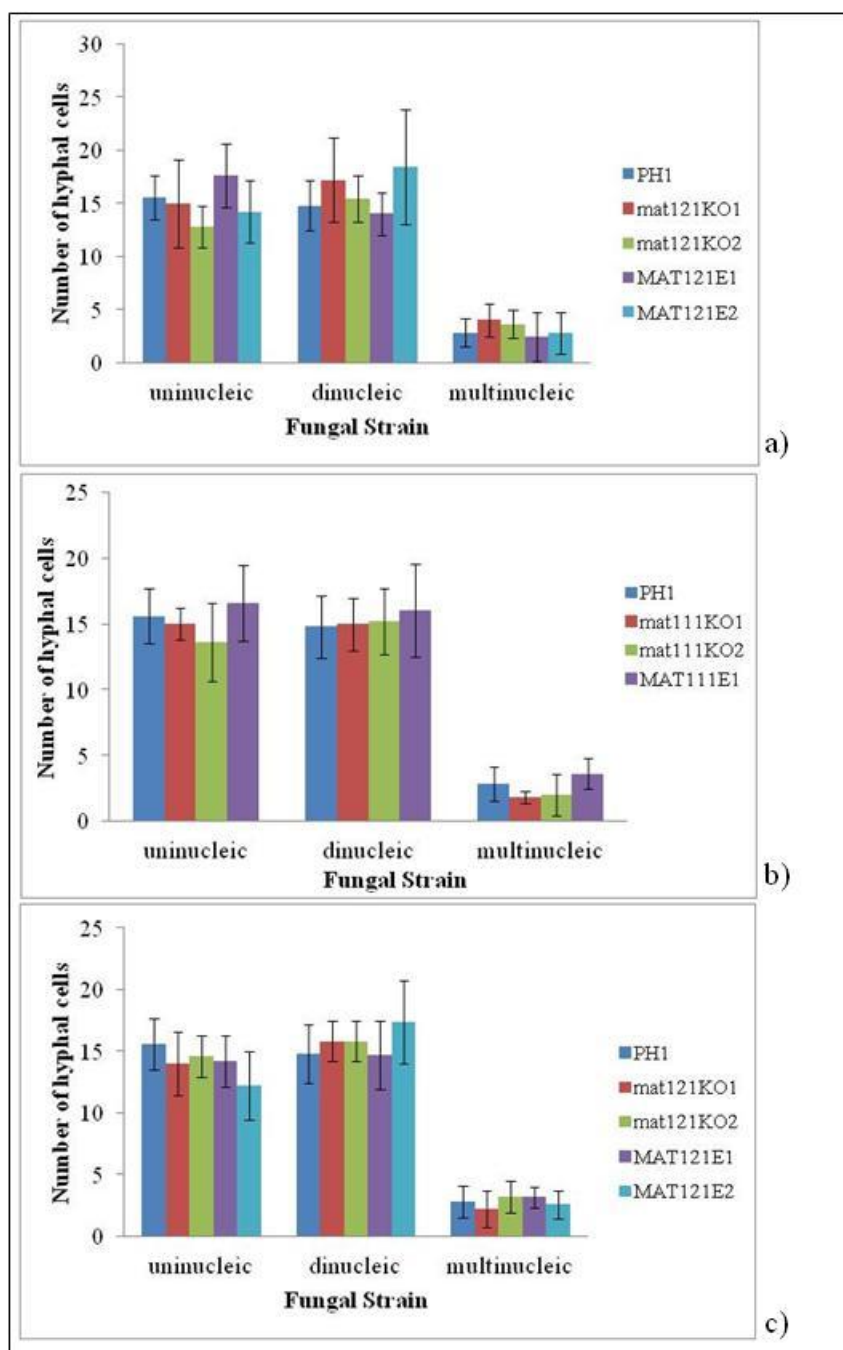
**Figure 5.4.** Sexual fertility phenotypes of MAT gene knockout (KO) mutants and ectopic (E) transformants. a1= *mat1*KO perithecial initials (40x); a2= *mat1*KO crushed perithecial initial (400x); b1= MAT1E fertile perithecia (40x); b2 = MAT1E crushed perithecium; c1= *mat1-1-1*KO perithecial initials (40x); c2= *mat1-1-1*KO crushed perithecial initial (400x); d1= MAT1-1-1E fertile perithecia (40x); d2 = MAT1-1-1E crushed perithecium; e1= *mat1-2-1*KO perithecial initials (40x); e2= *mat1-2-1*KO crushed perithecial initial (400x); f1= MAT1-2-1E fertile perithecia (40x); f2 = MAT1-2-1E crushed perithecium; g1= PH-1 wild type fertile perithecia; g2 = PH-1 WT crushed perithecium; h1= *mat1-1-1*KO x *mat121*KO heterothallic fertile perithecia (40x); h2 = *mat1-1-1*KO x *mat1-2-1*KO crushed perithecium. Black arrows indicate asci containing ascospores. White arrows indicate fertile perithecia containing asci with ascospores. Yellow arrows point to infertile perithecial initials that do not contain ascospores.



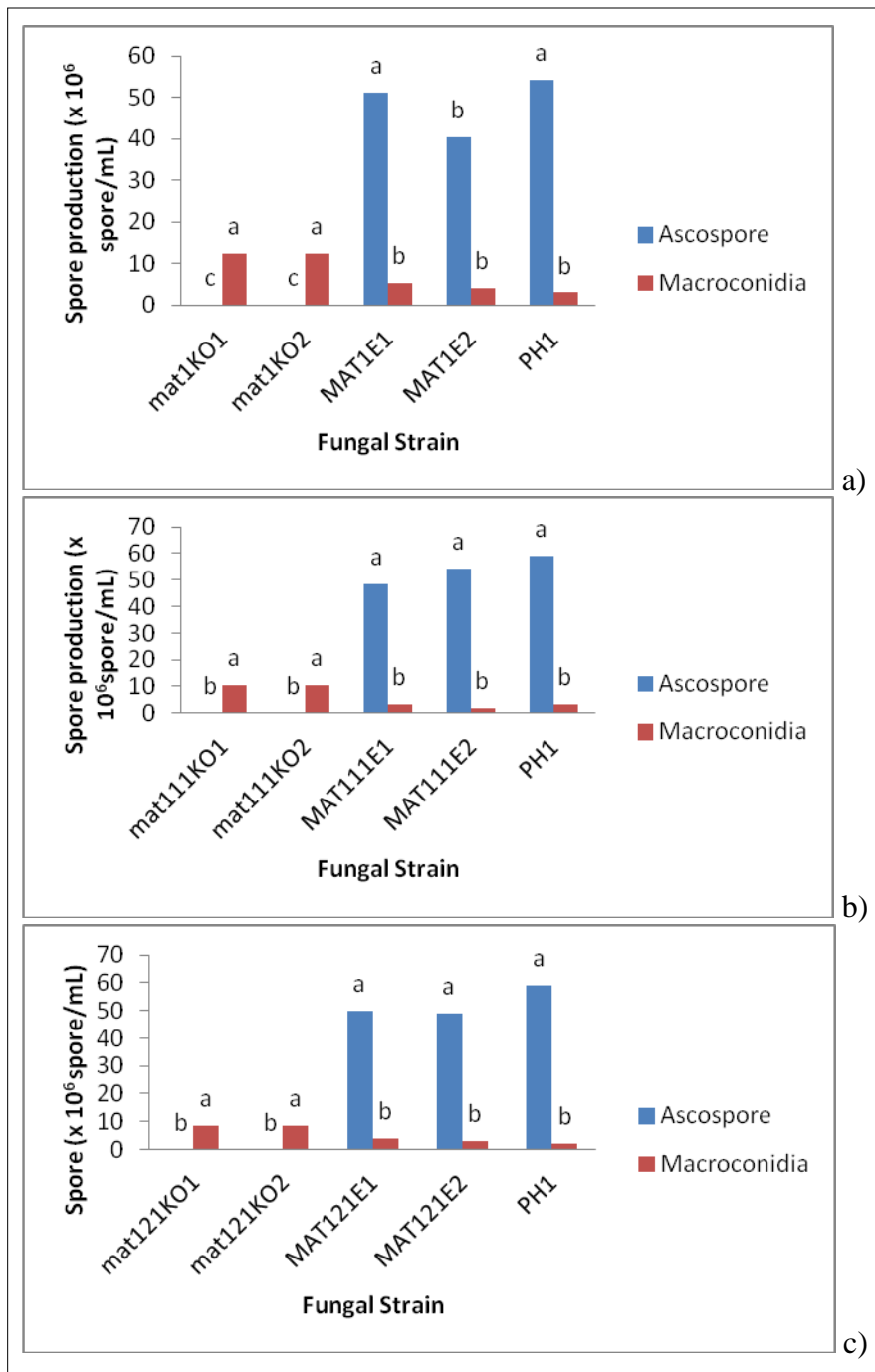
**Figure 5.5:** Effect of MAT1 (a), MAT1-1-1 (b) and MAT1-2-1 (c) deletion on the diameter of perithecia or perithecial initials in *G. zeae*. Different letters indicate a statistical difference at  $P = 0.05$ .



**Figure 5.6:** Micrograph of acridine orange (AO)-stained hyphae of a *G. zeae* mat1KO mutant strain (400 x). The arrows point to the specific hyphal types: white arrow (panel a and b), binucleate thick hyphae; yellow arrow (panel b), multinucleate thick hyphae; blue arrow (panel a), coiled hypha that will eventually produce perithecial initials; red arrow (panel b), thin mononucleate hyphae. The hyphae of other WT, ectopic, and KO strains appeared similar.

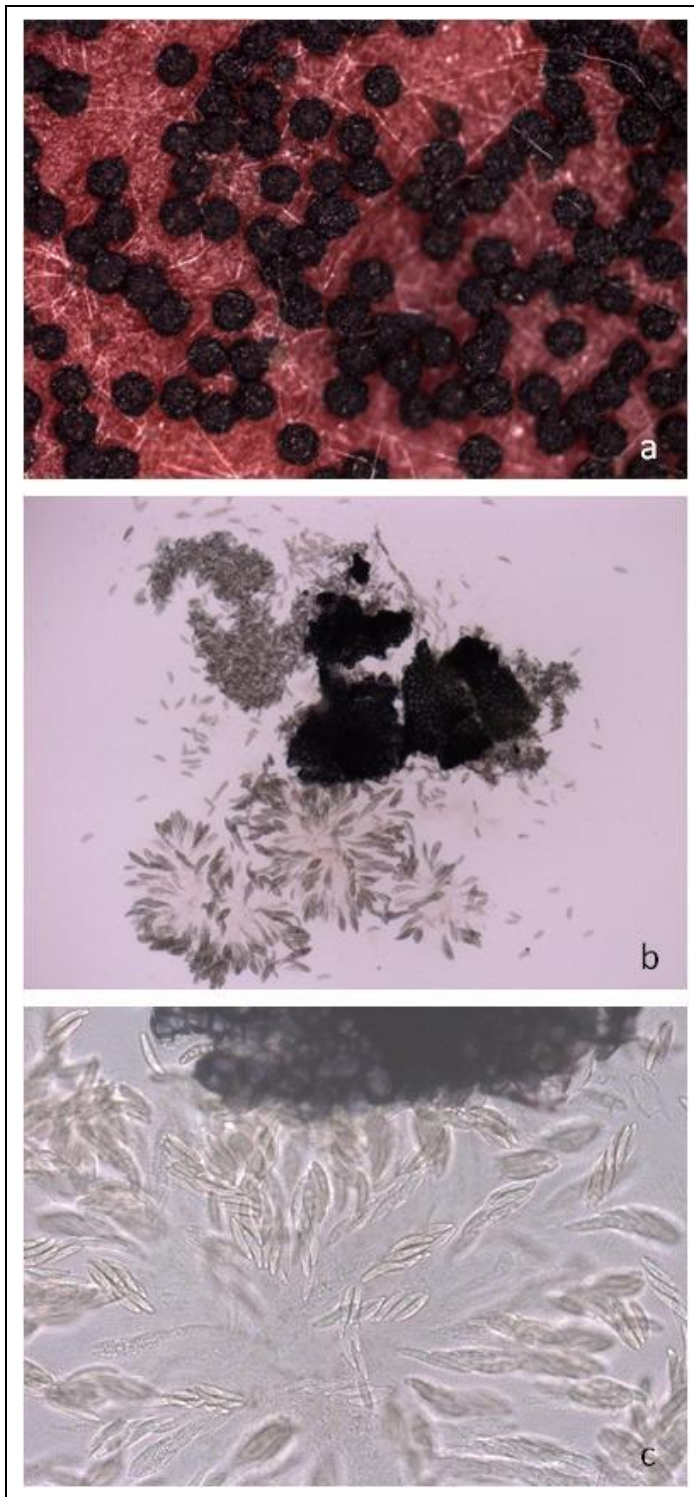


**Figure 5.7:** Effect of MAT1 (panel a), MAT1-1-1 (panel b), and MAT1-2-1 (panel c) deletion on the number of mono-, bi-, and multi-nucleate cells produced by each strain after 4 days of vegetative growth on carrot agar. The data points represent an average number of cells per field of view, determined by staining fragments of vegetative mycelia with acridine orange and visualizing with epifluorescent microscope at 400 x magnification.

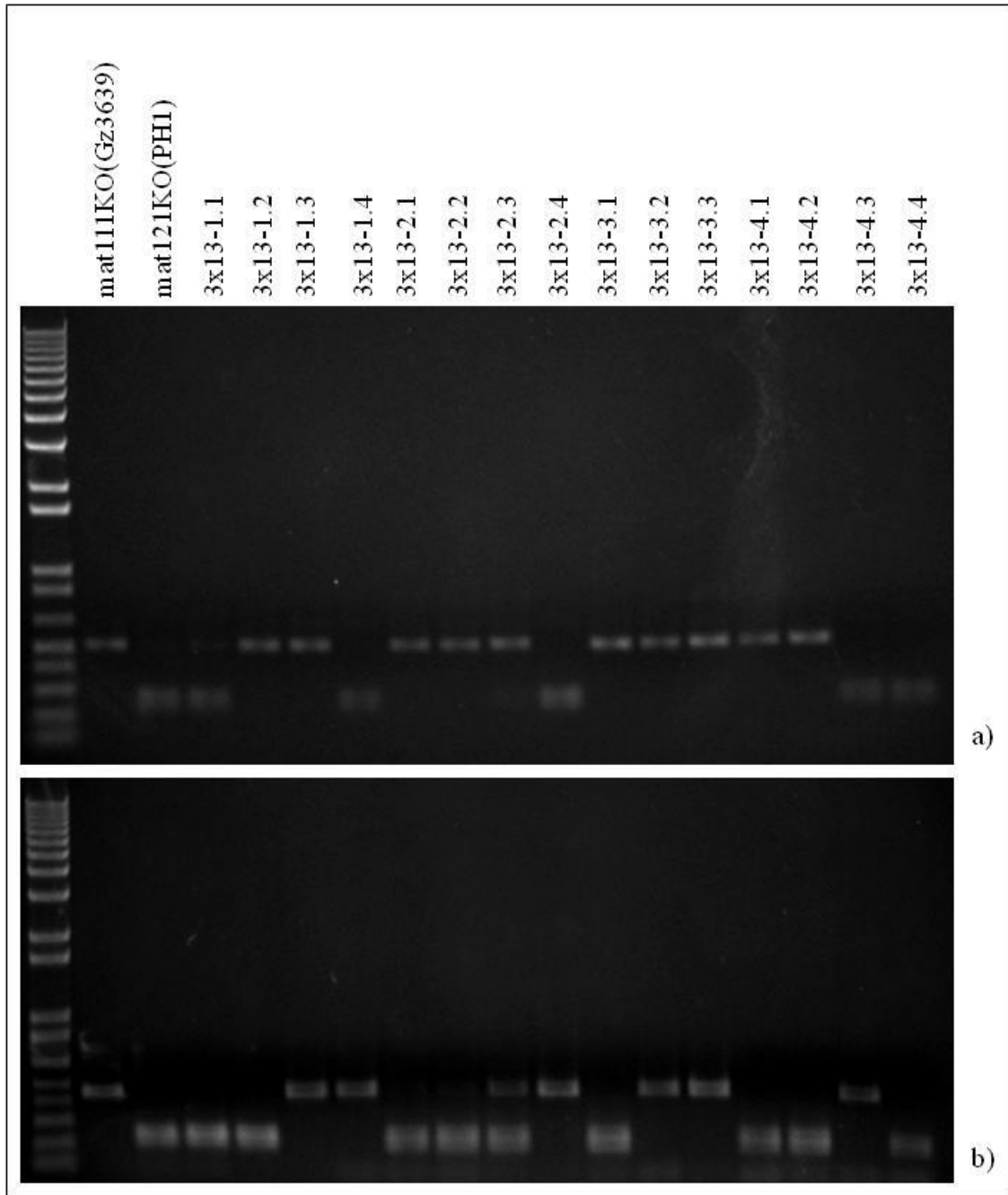


**Figure 5.8:** Effect of MAT1 a), MAT1-1-1 b), and MAT1-2-1 c) gene deletion on sexual(blue bars) and asexual (red bars) fecundity of *G. zeae*. Letters above the bars signify significant differences at  $P = 0.05$  associated with separate series, asexual(red) and sexual(blue)



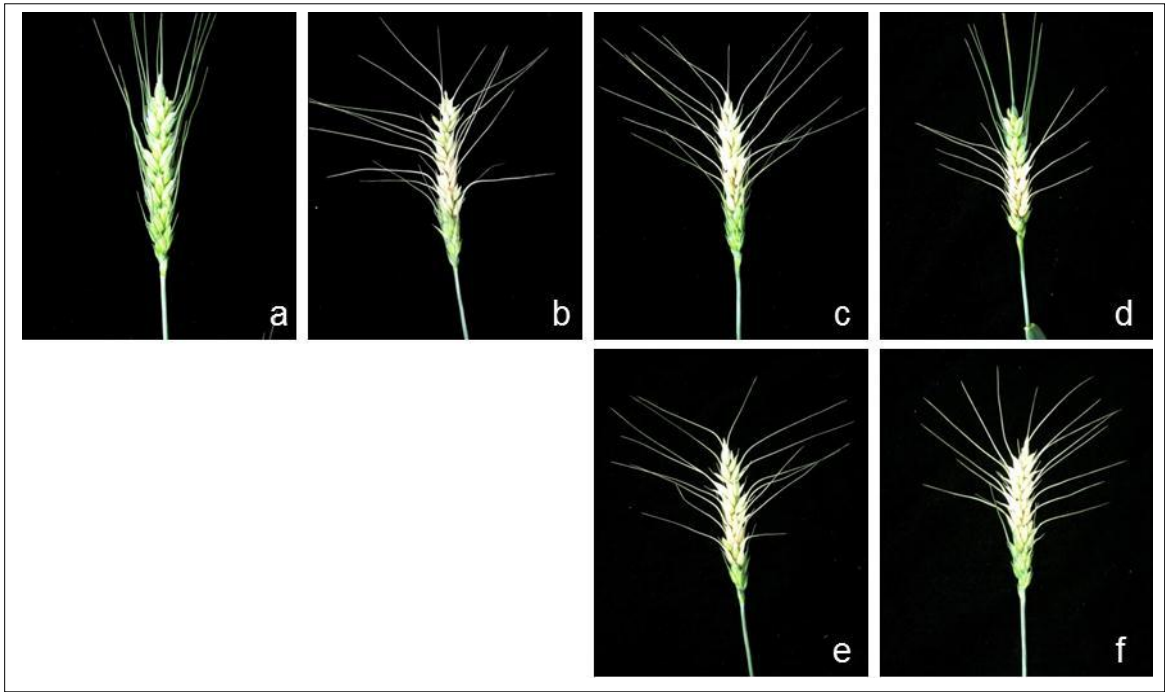


**Figure 5.9:** Fertile perithecia produced from a cross between mat1-1-1KO and mat1-2-1KO strains. A) Mature heterothallic perithecia, 40X; B) Crushed heterothallic perithecia, 100X; C) Mature asci with eight ascospores per ascus, 400X.

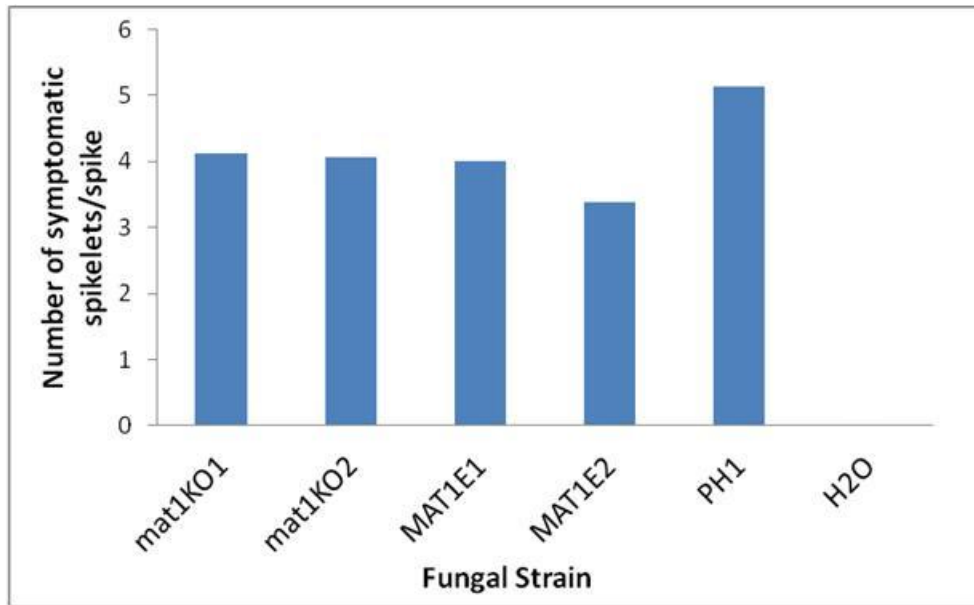


**Figure 5.10:** Segregation of CAPs markers, PstI2 (a) and EcoRI3 (b) among the progeny of a mat111KO(Gz3639) x mat121KO(PH1) cross derived from four randomly selected perithecia. Both CAPs markers segregated in 1:1 ratio with  $\chi^2 = 1.6667$ ,  $P=0.999$  for PstI2, and  $\chi^2 = 0.6$ ,  $P=0.999$  for EcoRI3 CAP.





**Figure 5.11:** The effect of the MAT1 locus deletion on the development of external FHB symptoms on the susceptible wheat variety Pioneer 2555. The images show representative FHB symptoms 7 days post-inoculation (dpi): a = H<sub>2</sub>O negative control, b = PH-1 wild type (WT), c = mat1KO1, d = mat1KO2, e = MAT1E1, f = MAT1E2.



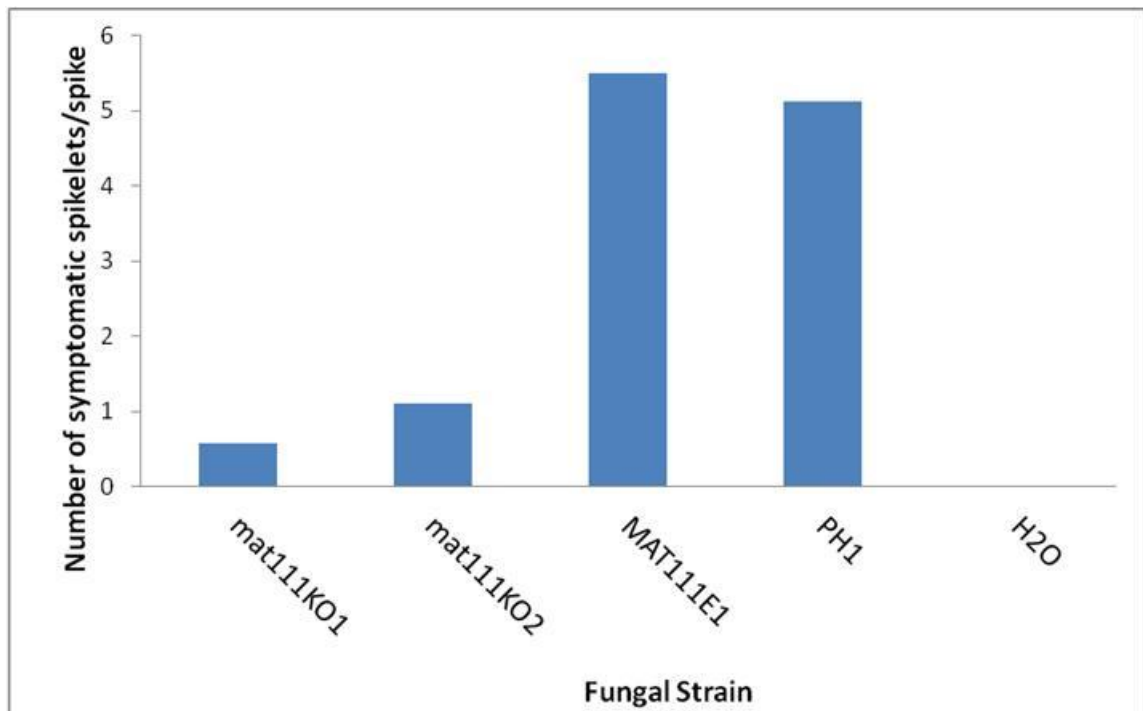
**Figure 5.12:** The effect of deletion of the MAT1 locus on the development of external FHB symptoms on the susceptible wheat variety Pioneer 2555 at 7 dpi.

**Table 5.1:** LSD matrix of pair-wise comparisons for the effect of fungal strain on the level of aggressiveness in the MAT1 locus deletion experiment. Numbers denoted in red are significantly different at  $P < 0.05$ .

i/j	H2O	mat1KO1	mat1KO2	MAT1E1	MAT1E2	PH-1
H2O		<.0001	<.0001	<.0001	<.0001	<.0001
mat1KO1	<.0001		0.9355	0.3329	0.8714	0.1977
mat1KO2	<.0001	0.9355		0.3745	0.9355	0.1715
MAT1E1	<.0001	0.3329	0.3745		0.4192	0.0258
MAT1E2	<.0001	0.8714	0.9355	0.4192		0.1479
PH-1	<.0001	0.1977	0.1715	0.0258	0.1479	



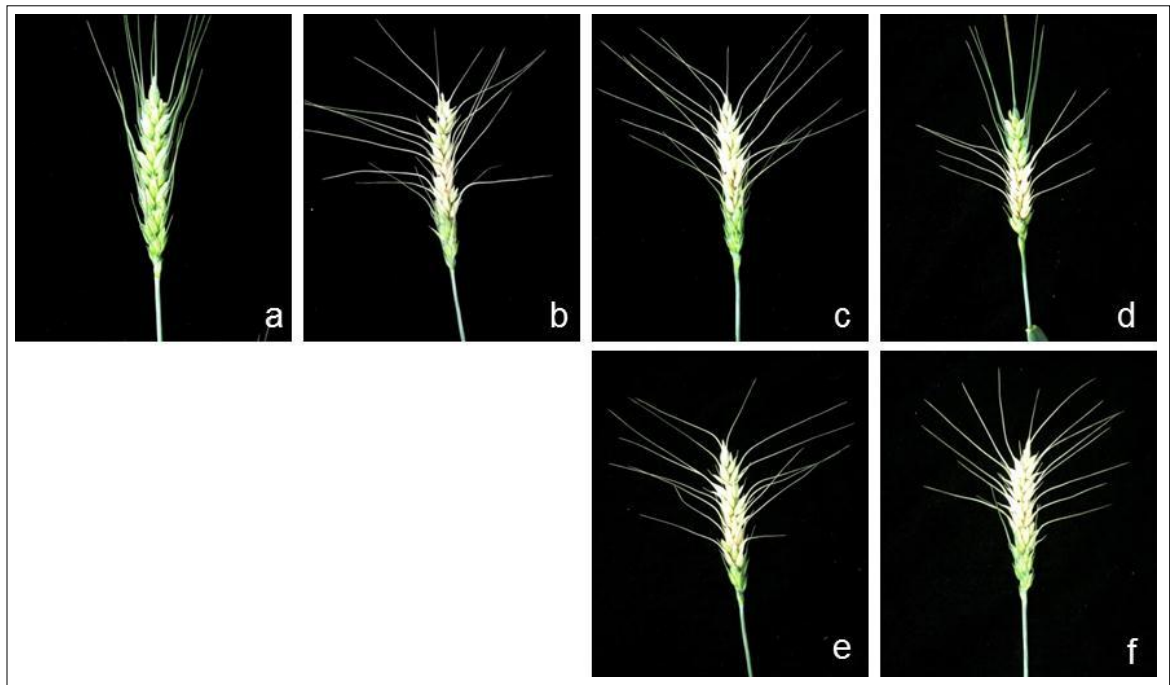
**Figure 5.13:** The effect of the MAT1-1-1 gene deletion on the development of external FHB symptoms on the susceptible wheat variety Pioneer 2555. The images show representative FHB symptoms 7 days post-inoculation (dpi): a = H<sub>2</sub>O negative control, b = PH-1 wild type (WT), c = mat111KO1, d = mat111KO2, e = MAT111E1.



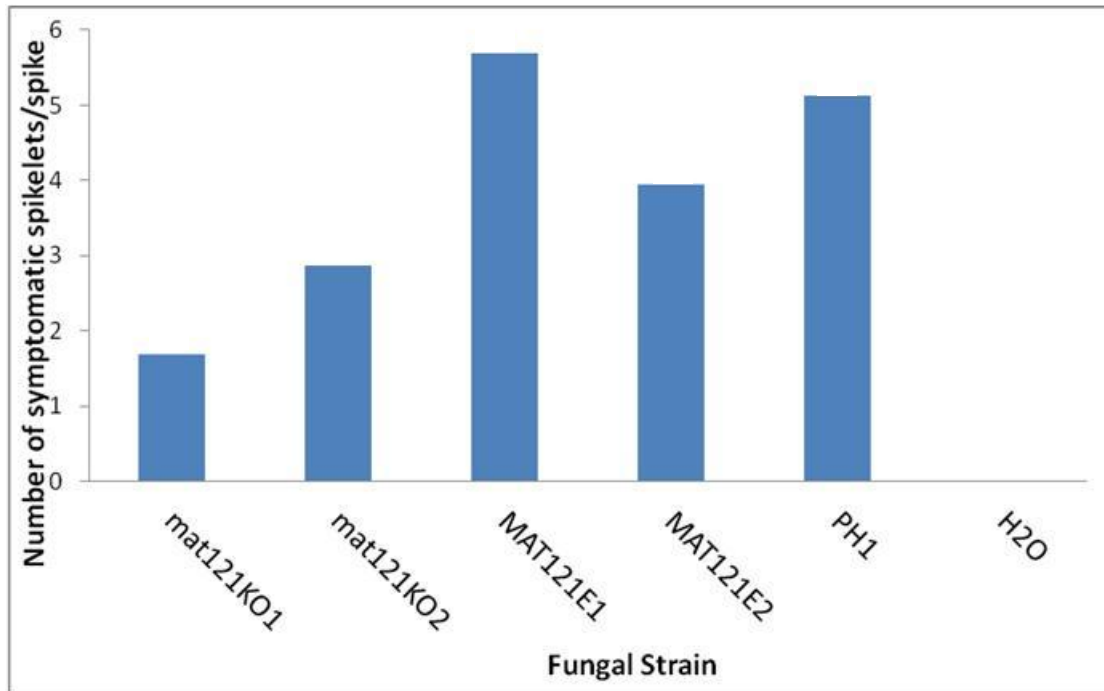
**Figure 5.14:** The effect of deletion of the MAT1-1-1 locus on the development of external FHB symptoms on the susceptible wheat variety Pioneer 2555 at 7 dpi.

**Table 5.2:** LSD matrix of pair-wise comparisons for the effect of fungal strain on the level of aggressiveness in the MAT1-1-1 locus deletion experiment. Numbers denoted in red are significantly different at  $P < 0.05$ .

i/j	H2O	mat111KO1	mat111KO2	MAT111E1	PH-1
H2O		0.3035	0.0505	<.0001	<.0001
mat111KO1	0.3035		0.3441	<.0001	<.0001
mat111KO2	0.0505	0.3441		<.0001	<.0001
MAT111E1	<.0001	<.0001	<.0001		0.5036
PH-1	<.0001	<.0001	<.0001	0.5036	



**Figure 5.15:** The effect of the MAT1-2-1 gene deletion on the development of external FHB symptoms on the susceptible wheat variety Pioneer 2555. The images show representative FHB symptoms 7 days post-inoculation (dpi): a = H<sub>2</sub>O negative control, b = PH-1 wild type (WT), c = mat121KO1, d = mat121KO2, e = MAT121E1, f = MAT121E2.

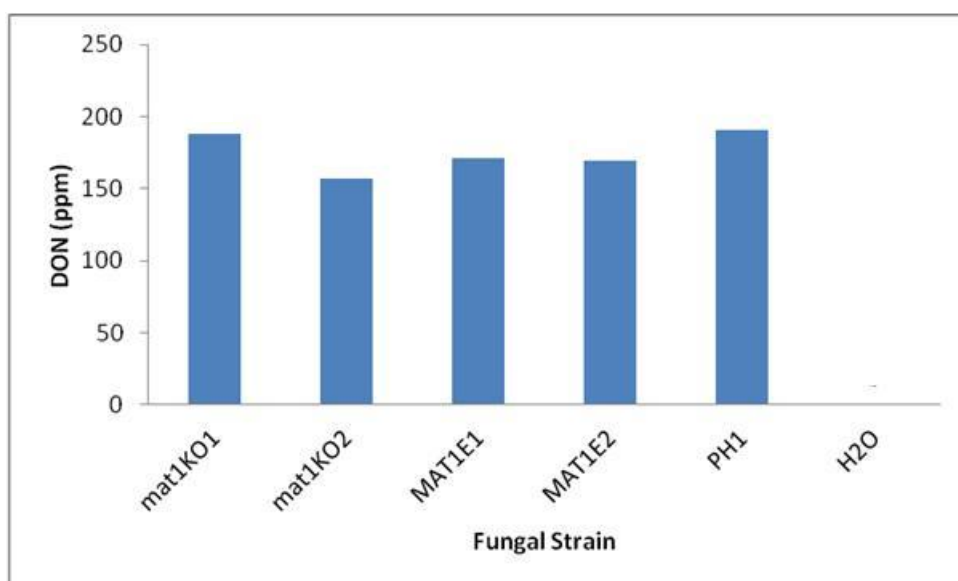


**Figure 5.16:** The effect of deletion of the MAT1-2-1 locus on the development of external FHB symptoms on the susceptible wheat variety Pioneer 2555 at 7 dpi.



**Table 5.3:** LSD matrix of pair-wise comparisons testing the effect of fungal strain on the level of aggressiveness in the MAT1-2-1 locus deletion experiment. Numbers denoted in red are significantly different at  $P < 0.05$ .

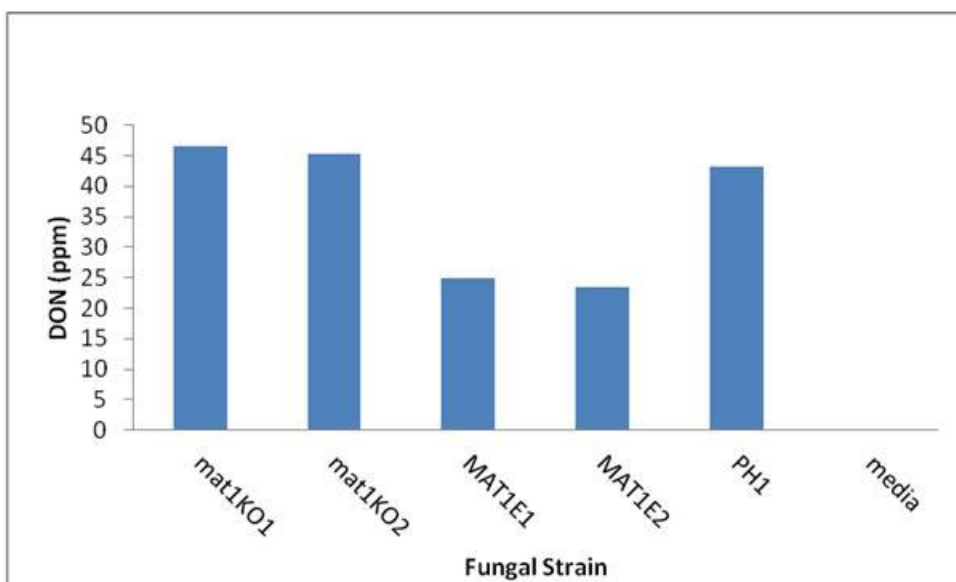
i/j	H2O	mat121KO1	MAT121E1	MAT121E2	mat121KO2	PH1
H2O		<.0001	<.0001	<.0001	0.0096	<.0001
mat121KO1	<.0001		<.0001	0.0985	0.0654	0.0007
MAT121E1	<.0001	<.0001		0.0086	<.0001	0.3899
MAT121E2	<.0001	0.0985	0.0086		0.0007	0.0654
mat121KO2	0.0096	0.0654	<.0001	0.0007		<.0001
PH1	<.0001	0.0007	0.3899	0.0654	<.0001	



**Figure 5.17:** Effect of deletion of the MAT1 locus on the average deoxynivalenol (DON) production *in planta* by *G. zeae* strains at 7dpi.

**Table 5.4:** LSD matrix of pair-wise comparisons for the effect of fungal strain on DON production *in planta* for the MAT1 deletion experiment. Numbers denoted in red are significantly different at  $P < 0.05$ .

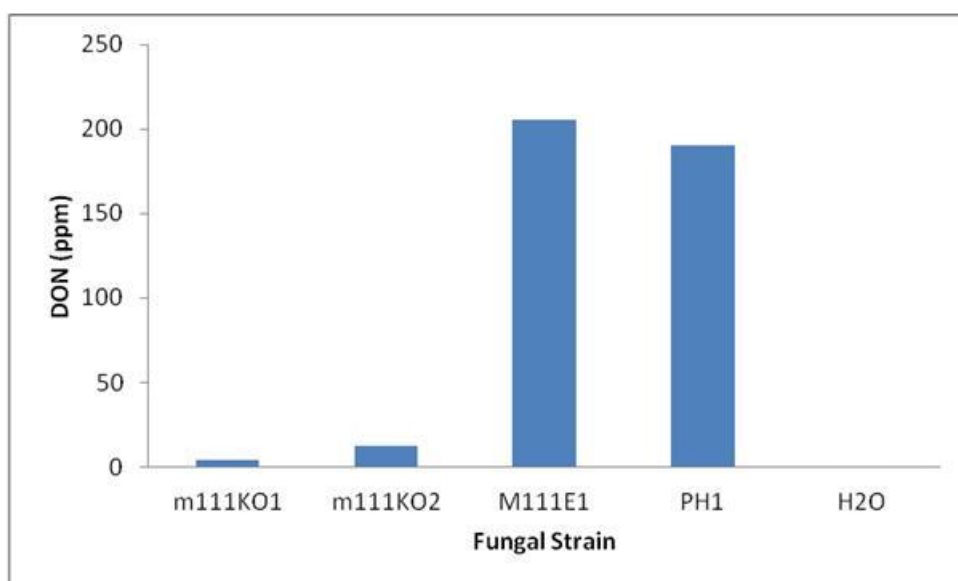
i/j	H2O	MAT1E1	MAT1E2	PH-1	mat1KO1	mat1KO2
H2O		<b>0.0001</b>	<b>0.0001</b>	<b>&lt;.0001</b>	<b>&lt;.0001</b>	<b>0.0004</b>
MAT1E1	<b>0.0001</b>		0.9674	0.6464	0.6849	0.7297
MAT1E2	<b>0.0001</b>	0.9674		0.6174	0.6551	0.7607
PH-1	<b>&lt;.0001</b>	0.6464	0.6174		0.9577	0.4219
mat1KO1	<b>&lt;.0001</b>	0.6849	0.6551	0.9577		0.453
mat1KO2	<b>0.0004</b>	0.7297	0.7607	0.4219	0.453	



**Figure 5.18:** Effect of deletion of the complete MAT1 locus on production of DON in media (2% GYEP) by *G. zeae*.

**Table 5.5:** LSD matrix of pair-wise comparisons for the effect of fungal strain on DON production in liquid media for the MAT1 deletion experiment.

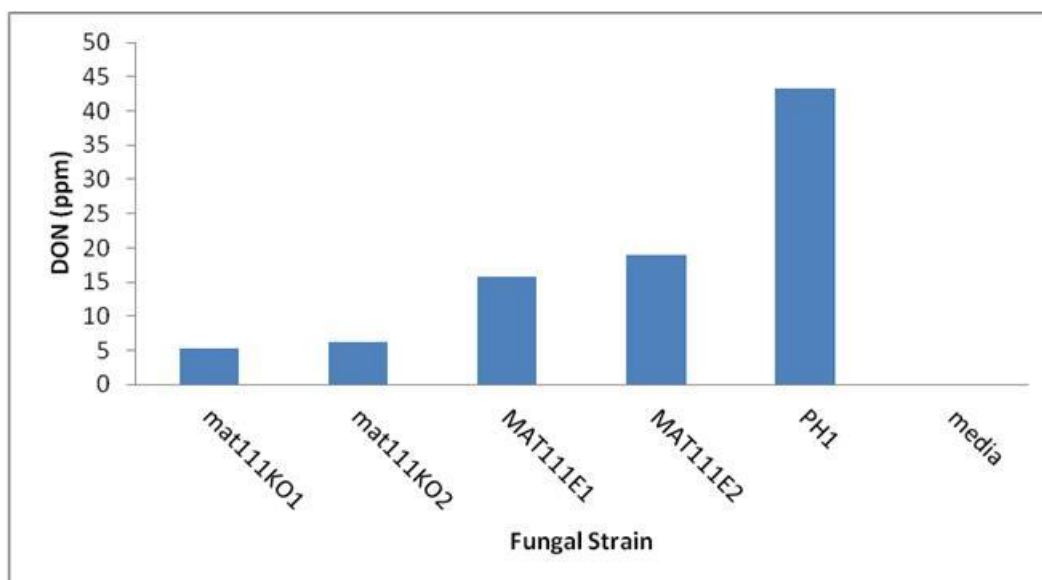
i/j	mat1KO1	mat1KO2	MAT1E1	MAT1E2	PH-1	media
mat1KO1		0.9622	0.4338	0.4014	0.8862	0.1335
mat1KO2	0.9622		0.4620	0.4283	0.931	0.1445
MAT1E1	0.4338	0.462		0.9546	0.4297	0.4174
MAT1E2	0.4014	0.4283	0.9546		0.3915	0.4470
PH-1	0.8862	0.9310	0.4297	0.3915		0.1111
media	0.1335	0.1445	0.4174	0.4470	0.1111	



**Figure 5.19:** Effect of deletion of the MAT1-1-1 locus on the average deoxynivalenol (DON) production *in planta* by *G. zeae* strains at 7dpi.

**Table 5.6:** LSD matrix of pair-wise comparisons for the effect of fungal strain on DON production *in planta* for the MAT1-1-1 deletion experiment. Numbers denoted in red are significantly different at  $P < 0.05$ .

i/j	H2O	MAT111E1	PH-1	mat111KO1	mat111KO2
H2O		<.0001	<.0001	0.8928	0.6519
MAT111E1	<.0001		0.5948	<.0001	<.0001
PH-1	<.0001	0.5948		<.0001	<.0001
mat111KO1	0.8928	<.0001	<.0001		0.7588
mat111KO2	0.6519	<.0001	<.0001	0.7588	

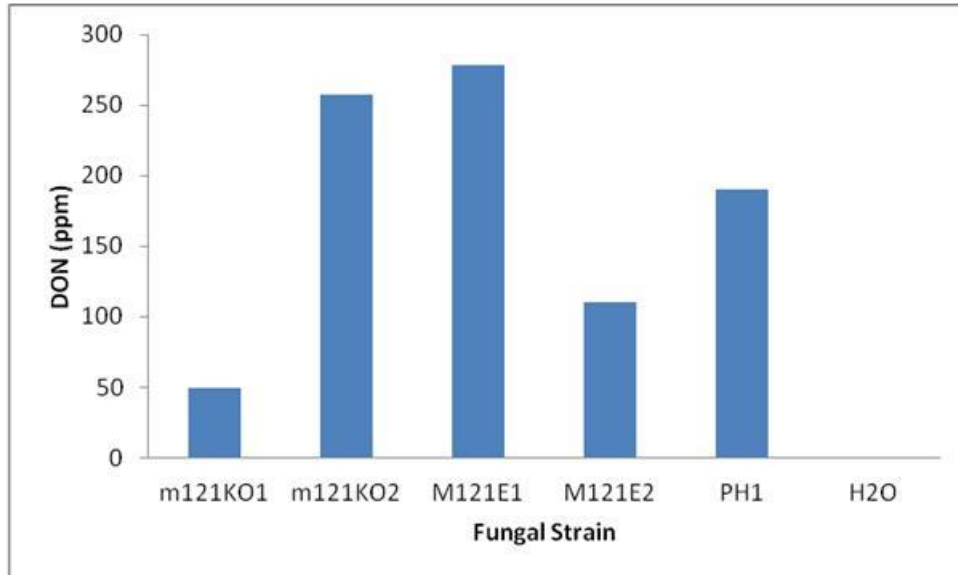


**Figure 5.20:** Effect of MAT1-1-1 deletion on production of DON in liquid media (2% GYEP) by *G. zeae*.



**Table 5.7:** LSD matrix of pair-wise comparisons for the effect of fungal strain on DON production in media for the MAT1-1-1 deletion experiment. Numbers denoted in red are significantly different at  $P < 0.05$ .

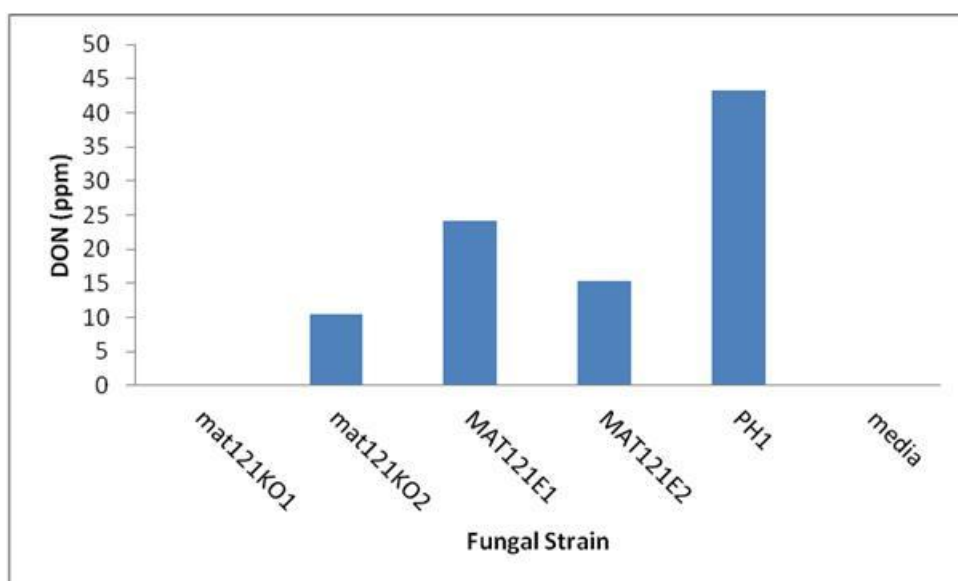
i/j	mat111KO1	mat111KO2	MAT111E1	MAT111E2	PH1	media
mat111KO1		0.9659	0.6252	0.5286	<b>0.0396</b>	0.8264
mat111KO2	0.9659		0.6557	0.5568	<b>0.0445</b>	0.7967
mat111E1	0.6252	0.6557		0.8869	0.1338	0.5121
mat111E2	0.5286	0.5568	0.8869		0.1822	0.4343
PH1	<b>0.0396</b>	<b>0.0445</b>	0.1338	0.1822		<b>0.0438</b>
media	0.8264	0.7967	0.5121	0.4343	<b>0.0438</b>	



**Figure 5.21:** Effect of deletion of the MAT1-2-1 locus on the average deoxynivalenol (DON) production *in planta* by *G. zeae* strains at 7dpi.

**Table 5.8:** LSD matrix of pair-wise comparisons for the effect of fungal strain on DON production *in planta* for the MAT1-2-1 deletion experiment. Numbers denoted in red are significantly different at  $P < 0.05$ .

i/j	H2O	MAT121E1	MAT121E2	PH1	mat121KO1	mat121KO2
H2O		<.0001	0.0039	<.0001	0.1901	<.0001
MAT121E1	<.0001		<.0001	0.021	<.0001	0.5814
MAT121E2	0.0039	<.0001		0.0353	0.1038	0.0002
PH1	<.0001	0.021	0.0353		0.0003	0.0758
mat121KO1	0.1901	<.0001	0.1038	0.0003		<.0001
mat121KO2	<.0001	0.5814	0.0002	0.0758	<.0001	



**Figure 5.22:** Effect of MAT1-2-1 deletion on production of DON in liquid media (2% GYEP) by *G. zeae*.

**Table 5.9:** LSD matrix of pair-wise comparisons for the effect of fungal strain on DON production in media for the MAT1-2-1 deletion experiment. Numbers denoted in red are significantly different at  $P < 0.05$ .

i/j	mat121KO1	mat121KO2	MAT121E1	MAT121E2	PH1	media
mat121KO1		0.6471	0.2732	0.4882	<b>0.0218</b>	0.9974
mat121KO2	0.6471		0.5422	0.8293	0.0911	0.678
MAT121E1	0.2732	0.5422		0.6843	0.3025	0.325
MAT121E2	0.4882	0.8293	0.6843		0.1322	0.5329
PH1	<b>0.0218</b>	0.0911	0.3025	0.1322		<b>0.047</b>
media	0.9974	0.678	0.325	0.5329	<b>0.047</b>	

## CHAPTER 6

### Summary

#### Where were we then?

When I joined the Vaillancourt laboratory as a graduate student, I chose to work on *Gibberella zeae* because it causes Fusarium Head Blight, one of the most important diseases affecting one of the most important crops (wheat) worldwide. (Doohan, Brennan, and Cooke, 2003; Miedaner, 1997; Parry, Jenkinson, and McLeod, 1995). FHB had become particularly damaging in North America beginning during the 1990s, with major losses in grain yield and grain quality. Mycotoxins produced by *G. zeae* are very harmful to human and animal health. As a result of the FHB epidemics and related financial losses, many small family farms in the upper Midwest disappeared (Windels, 2000). Thus, FHB didn't have only an agronomic, but also a cultural effect on the local communities that especially touched me, having grown up on a small farm myself. I knew the challenges that faced me, as the first person in my laboratory to work on this pathosystem, but the potential rewards made it worthwhile to me.

Integrated disease management practices incorporating the use of moderate to highly resistant wheat varieties, timely application of fungicides with high efficacy, and appropriate agronomic practices that reduce survival and overwintering of pathogen on plant debris, have the best effect on controlling the potential for the onset of an FHB epidemic (McMullen et al., 2008). However, even with the availability of these tools, the disease has continued to cause devastation.

Currently, relatively limited sources of resistance are being used in wheat breeding programs, with a majority related to the resistance found in the Chinese wheat variety Sumai 3 (Liu and Anderson, 2003; Liu et al., 2006; Shen, Francki, and Ohm, 2006; Yu et al., 2008; Voss et al., 2010). With such a narrow genetic base, it becomes very important to understand the potential that the pathogen has for overcoming this resistance through genetic changes. When I began my work, I found that relatively little was known about this topic, although it was becoming a very active area of research.

In North America *G. zeae* (anamorph *F. graminearum*) is the primary causal agent of FHB (Goswami and Kistler, 2004). This fungus is a homothallic ascomycete that is able

to out-cross under laboratory conditions (Bowden and Leslie, 1999). Based on the genetic diversity present in field populations, it was also believed to out-cross in nature (Miedaner et al, 2001; Gale et al., 2002; Schmale et al., 2006; Gale et al., 2007). However, the frequency of out-crossing in the field has never been measured. The frequency of out-crossing will determine, in part, how quickly a genetic variant can spread through the population. It is reasonable to expect that the potential for selection of genetic variants will be high if they are related to pathogen survival and offer a competitive advantage to the pathogen. I decided that what we needed was a better understanding of the potential for out-crossing to create new genotypes that offered a selective advantage, and better ways to track out-crossing and genetic diversity in the field.

### **Where are we now?**

Since I began my research on *Fusarium graminearum*, a lot has changed. Thanks to an influx of financial support (thank you U.S. Wheat and Barley Scab Initiative!) and a lot of research activity in the *Fusarium* community, progress has been made (although there is still far to go). After doing a lot of reading, and making many beginners mistakes, I think that I have been able to add some useful information and to be a part of that progress.

**Question 1:** What is the potential for out-crossing to create new genotypes that offer a selective advantage?

In order to answer that question I crossed the two most commonly used *F. graminearum* strains PH-1 and Gz3639. I think that just making a direct comparison of the two for the first time is an important contribution to the community. Some of the “conventional wisdom” about these two strains appears to be inaccurate, but two things turned out to be true: Gz3639 is more toxigenic, and PH-1 is more fertile. The advantage of choosing these parental strains for my study is that they have the best available genetic and genomic resources. The availability of a list of SNPs helped in creation of CAPs markers I used to identify recombinant perithecia and derive a F1 population. It also makes it possible to map and ultimately identify segregating genes of interest in the cross, a project that is currently underway.

The results of my crossing experiment were very exciting, and showed that out-crossing among even these phenotypically-similar, lineage 7, 15-ADON strains had the potential to produce highly aggressive, highly toxigenic progeny. None of the progeny “broke” two sources of resistance that they were tested on, suggesting that perhaps this was beyond the genetic potential of these two parental strains. Last year, after I had already progressed substantially in my work, an article was published that reported similar results, but with crosses of field isolates that varied in their aggressiveness to wheat (Voss et al. 2010). However, because I did my crosses with well-characterized genome-sequenced lab strains, my work has more potential in the long run to facilitate identification of individual genes involved in aggressiveness.

What was most surprising to me was that even selfed perithecia produced progeny that were more aggressive than their parent (though to a lesser degree than the out-crossed perithecia), hinting at a previously unsuspected mechanism by which sexual reproduction can contribute to phenotypic diversity. This is really exciting, and more research is needed to discover the mechanism of that variation, its extent in field strains as well as in laboratory strains, and its heritability and thus potential for selection in the field. My preliminary work suggested that DNA methylation could be involved, but I did not confirm this. However, I did develop some tools that could be used in future studies of the role of methylation. For more information on this part of my study, I refer you to Appendix 3 of this Dissertation.

Various hints in the literature suggested an intriguing hypothesis that the MATing type genes themselves, which control fertility in *G. zeae*, might be directly involved in pathogenicity. I wanted to test this hypothesis using a molecular approach, but first I needed a full set of MAT mutant strains and appropriate ectopic controls, since previously published strains were no longer available. I tested two different KO strategies, intact marker (IM) and split marker (SM), but I found there was little difference between them and I was able to generate MAT1, MAT1-1-1, and MAT1-2-1 KO strains by both strategies. However, I did find that the IM protocol produced suitable ectopic control strains whereas the SM protocol did not.



Results of my experiment showed that deletion of the entire MAT1 locus had no effect on pathogenicity or on mycotoxin production. However, very intriguingly, the deletion of either MAT1-1-1 or MAT1-2-1 alone had a negative effect, which was more pronounced with deletion of MAT1-1-1. This suggests that presence of either MAT protein alone, in the absence of the other, is detrimental to pathogenicity. The experiment was a very large one that included fifteen replicates for each treatment, and I have a lot of confidence in the results, but unfortunately I was not able to replicate it due to an epidemic of wheat curl mites in the greenhouse. We have since conducted a preliminary aggressiveness experiment in corn, where *G. zea* causes an important stalk rot disease, and the results of this experiment also showed a significant reduction in aggressiveness of the MAT 1-1-1 KO strain. More work is needed to confirm these results, but so far the hypothesis that the MATing type genes may have a direct effect on pathogenicity seems to be supported.

**Question 2.** How can we track out-crossing and genetic diversity in the field?

In order to address this question I took advantage of the publically available PH-1 genome sequence. With the help of Dr. Mark Farman, I developed a novel set of RFLP probes useful for study of population diversity, and also applicable as genetic markers so that they can be used to track out-crossing in the field.

I tested these repetitive RFLP probes on a local population of *G. zea*, which has not been done before, and I was able to show that there was a lot of genetic diversity in these populations. I think these markers could be useful for the U.K. wheat-breeding team in the future, to track the diversity of their test strains. Very interestingly, I identified two species of *Fusarium* from blighted wheat heads from Owensboro that are not *F. graminearum* strains. One is a species more often associated with barley head blight, and the other may be a previously undescribed species. More work will need to be done to determine how much of a threat these novel species might be to Kentucky wheat crops. The tools I have developed will help in this work.

### **And now, what?**

According to NPR, in November 2011 the global human population reached 7 billion. How will we meet the food requirements of this ever-growing world population? Our success will depend, as it always has, on agricultural research.

For the past 6 + years of my life I have immersed myself in the life of *G. zeae*, a tiny fungus with a big impact on food commodities produced from grain crops (wheat, maize, small grains). Equal to the reality that the sun will come up tomorrow, it is guaranteed that *G. zeae* will evolve under the pressure of strategies employed to control its survival and the deleterious effects it has on wheat, wheat production, and global food supply. We can either choose to look at this inevitable evolution with fear and uncertainty about what is to come, or we can accept it as a driving force behind our scientific thought and progress.

To me, it is an amazing thing to sit behind a computer and delve into the scientific data generated on the subject of *G.zeae*, and realize how little we actually know and how much there is still to uncover. With the completion of this dissertation, I am temporarily "abandoning" my research project, but I hope that my work added a morsel of understanding, and created a strong foundation for another graduate student to come and develop the same love and appreciation for the wonders of life, even if that be the life of a "pathogen".

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## APPENDIX I

### **Genetic and morphological characterization of a MAT1-1-1 strain provided to us by Dr. Frances Trail (Michigan State University).**

For a part of my work (Chapters 2 and 5) I used a MAT1-1-1 mutant strain that was provided to me by Dr. Frances Trail of Michigan State University. This is the only MAT KO strain that I was able to obtain from members of the Fusarium community, since apparently all the other published strains had been lost. Dr. Trail was not the one that had created the mutant she sent me, and she was somewhat unsure of its history, although she believed that it had been produced in Dr. John Leslie's laboratory at Kansas State University in the Gz3639 background. Dr. Leslie did not recollect the strain, but thought that it could well have been produced in his laboratory. Neither Dr. Trail nor Dr. Leslie had ectopic controls for this strain

It was for these reasons that I created a new set of MAT KO mutant strains in the PH-1 background. However, a MAT KO mutant strain in a different WT background would be useful, hence I did significant work to clarify the identity of this  $\Delta$ mat1-1-1 KO strain.

### **Results and Discussion:**

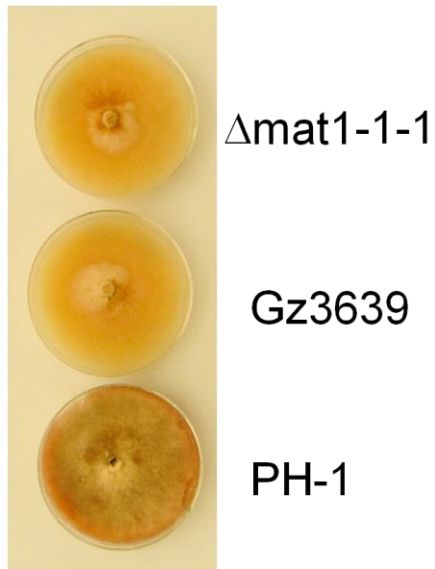
The results of my analysis were consistent with the hypothesis that the strain I obtained from Dr. Trail is indeed a  $\Delta$ mat1-1-1 KO of Gz3639.

It's appearance overall was very similar to Gz3639, and very different from PH-1. (Figure A1.1). A RAPD fingerprint analysis of DNA isolated from the strain showed that the pattern of this mutant strain is identical with the pattern of Gz3639 (Figure A1.2). The CAPs and RFLP markers also produced patterns that were identical with the Gz3639 WT (Chapter 2 of this Dissertation). I sent some DNA from the strain to Dr. Leslie at KSU and he performed AFLP analysis that confirmed that this strain was indeed in the Gz3639 genetic background (John Leslie, personal communication).

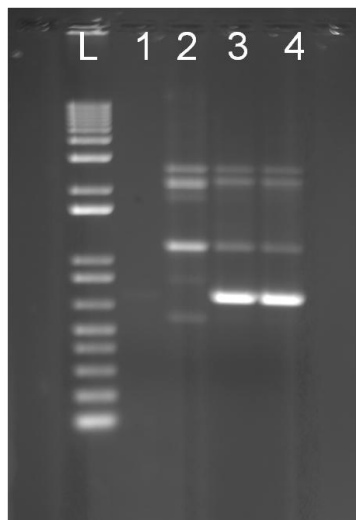
The strain was resistant to hygromycin (Figure A1.3a), and a fragment of the hygromycin phosphotransferase (*hph*) selectable marker gene could be amplified from it (Figure A1.3b), consistent with it having been transformed with *hph*.

The mutant strain had the phenotype of a MAT KO, and didn't produce perithecia on carrot agar (CA), while the control WT strains, PH-1 and Gz3639, did (Figure A1.4). A spore sample harvested from 28-day old CA Petri plates shows that the putative  $\Delta$ mat1-1-1 KO produces only macroconidia, whereas WT Gz3639 produces mostly ascospores and a very few macroconidia (Figure A1.5). The mutant strain was compatible with the MAT1-2 -1 KO of PH-1, consistent with its identity as a MAT1-1-1 KO (Chapters 2 and 5 of this dissertation).

## RESULTS:

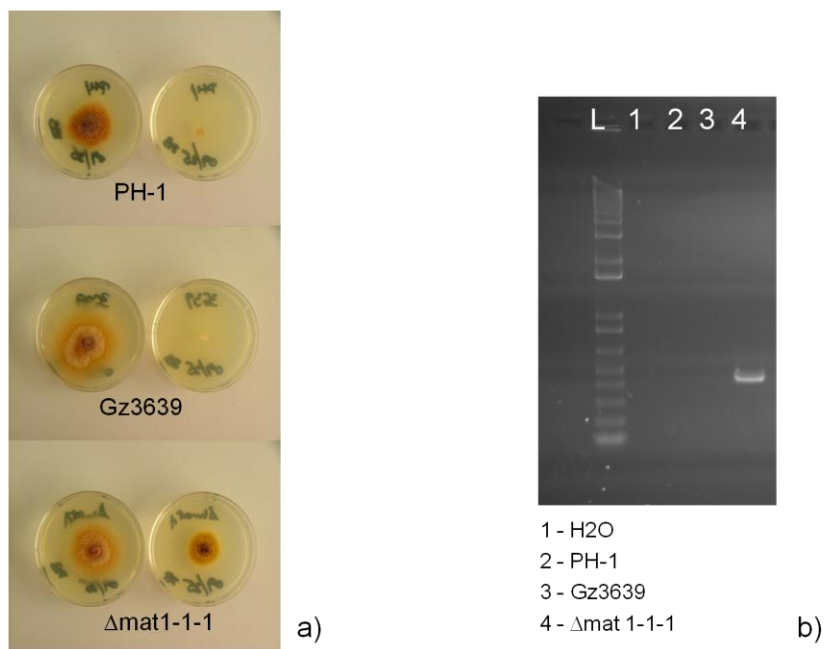


**Figure A1.1.** Comparison of colony morphology (seven days growth on PDA media) between PH-1, Gz3637 and putative  $\Delta\text{mat1-1-1}$  KO made in Gz3639 background.

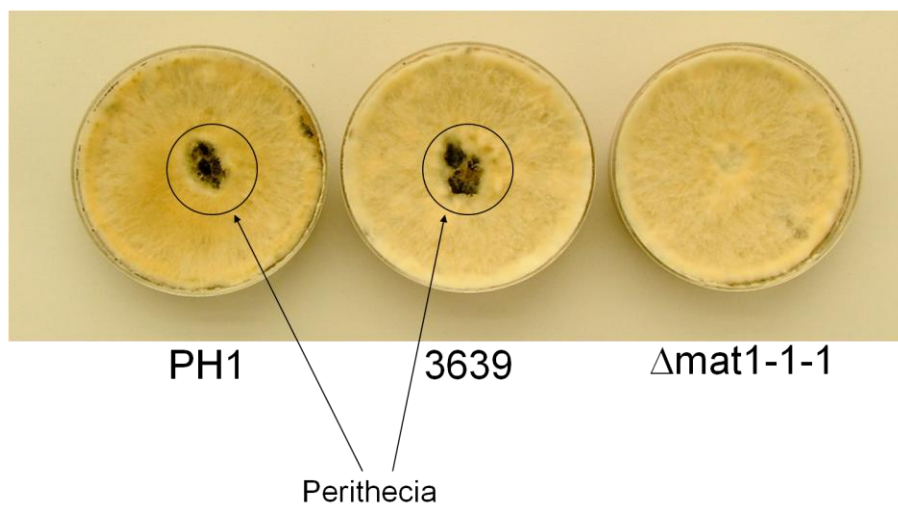


- 1- H<sub>2</sub>O
- 2- PH-1
- 3 - Gz3639
- 4 -  $\Delta\text{mat1-1-1}$

**Figure A1.2.** Comparing the RAPD pattern between PH-1, Gz3639 and  $\Delta\text{mat1-1-1}$  KO using OPA19 oligomer.

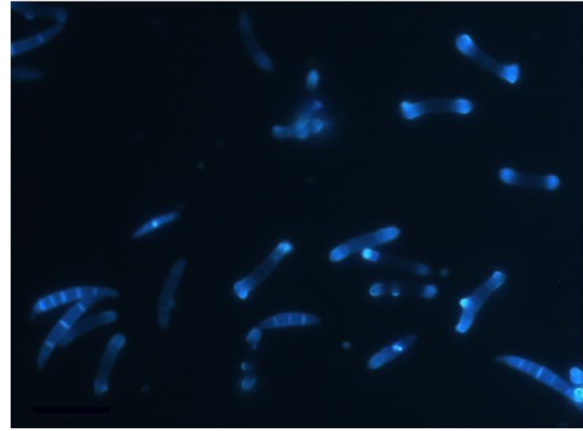
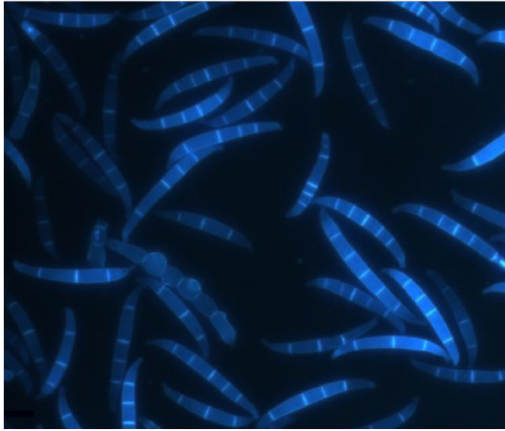


**Figure A1.3.** Confirmation of resistance to hygromycin present in  $\Delta$ mat1-1-1 KO by growth on PDA + hygromycin (a), and PCR confirmation of the presence of hygromycin resistance gene in the  $\Delta$ mat1-1-1 KO strain.



**Figure A1.4.** The perithecia induction on carrot agar (CA) media (28 day old)





$\Delta$  mat111 macroconidia > only macroconidia (a) Mixture of ascospores and macroconidia from Gz3639 (b)

**Figure A1.5.** Spores produced by  $\Delta$ mat1-1-1 KO (a) and Gz3639 (b) on the 28-day old CA media.

## APPENDIX II

**Sequences for the *Fusarium* sp. strains isolated from FHB symptomatic wheat heads collected in Owensboro, KY and Hatfield, IN.**

a) EF1 sequences for the *Fusarium* sp. strains isolated from FHB symptomatic wheat heads collected in Owensboro, KY and Hatfield, IN.

These sequences were generated by Dr. Todd Ward, USDA, Peoria, IL to compare the Owensboro, KY and Hatfield, IN strains based on their EF1 sequences.

**>25797(+)**

TGTTCTTGATGAAATCACGGTGACCGGGAGCGTCTGATAGACATGTTAGT  
ATGAGAATGTGATGACAGCAGTGGTGACAACATACCAATGACGGTGACAT  
AGTAGCGAGGAGTCTCGAACTTCCAGAGGGCGATATCAATGGTGATACCA  
CGCTCACGCTCGGCTTTGAGCTTGTCAAGAACCCAGGCGTACTTGAAGGA  
ACCCTTACCGAGCTCGGCGGCTTCCTATTGACAGGTGGTTAGTGACTGGT  
TGACACGTGATGATGAGCGCCCAGGGAATGGGTTTGTGGGAAGAGGGCAA  
ACGCTGTGCTCGAGTGGCGGGGTATGAGCCCCACCAGGGGAAAAAATT  
ACGACAAAGCCGCAAAATTTTTGACCTCGAGCGGGGTAACATGCGCGTAT  
CGAGTCGTGCTGTGAGGGCGATTTCGAATGATATTTTCGAAAGGGAAAAGGG  
CGCGCGATCGAGGAAAATGAGACCAACCTTCTCGAACTTCTCGATGGTTC  
GCTTGTGATACCAACCGCACTGGTAGATCAAGTGACCGGTCTATCAAAGT  
ATGTCAGCACATTGGAAATTTGAACTACCCCGCCAAGTGTCGGCGGGGTT  
GGGATGCGGTGGTACTCACAGTGGTCGACTTGCCAGAGTCGACGTGGCCG  
ATGACGACGACGTTAAGGTG

**> OKY2-2.1B2 (Fg032\_Ky2.1) (*F. graminearum*)**

GTTCTTGATGAAATCACGGTGACCGGGAGCGTCTGATAGCCATGTTAGTA  
TGAGAATGTGATGACAGCAGTGGTGACAACATACCAATGACGGTGACATA  
GTAGCGAGGAGTCTCGAACTTCCAGAGGGCGATATCAATGGTGATACCAC  
GCTCACGCTCGGCTTTGAGCTTGTCAAGAACCCAGGCGTACTTGAAGGAA  
CCCTTACCGAGCTCGGCGGCTTCCTATTGACAGGTGGTTAGTGACTGGTT  
GACACGTGATGATGAGCGCCCAGGGAATGGTTTGTGGGAAGAGGGCAGAC  
GCCTGTGCTCGAGTGGCGGGGTATGAGCCCCACCAGGGGAAAAAATTACG  
ACAAAGCCGCAAAATTTTTGACCTCGAGCGGGGTAACAGGCGCGTATCGA  
GTCGTGCTGTGAGGGCGATTTCGAATGATATTTTCGAAAGGGAAAAGGGCGC  
GCGATCGAGGAAAATGAGACCAACCTTCTCGAACTTCTCGATGGTTCGCT  
TGTCGATACCAACCGCACTGGTAGATCAAGTGACCGGTCTATCAAAGTATG  
TCAGCACATTGGAAATTTGAACTACCCCGCCAAGTGTCGGCGGGGTTGG  
GATGCGGTGGTACTCACAGTGGTCGACTTGCCAGAGTCGACGTGGCCGAT  
GACGACGACGTTAAGGTGAG

**> OKY2-2.1B3 (Fg033\_Ky2.1) (*F. tricinctum* NRRL 36147, MLST2-a)**

CTCACCTTAACGTCGTCGTCATCGGCCACGTCGACTCTGGCAAGTCGACC  
ACTGTAAGTACAACCAACAGCGGGTTGCTTATCTGCACTCGGAATCCGCC  
AAACCTGGCAGGGTATCACCAAAACATCTTGCTAACTTTTGACAGACCGG

TCACCTTGATCTACCAGTGCGGGTGGTATCGACAAGCGAACCATCGAGAAGT  
TCGAGAAGGTTAGTCAATATCCCTTCGATTACGCGCGCTCCCATCGATT  
CCACGATTCGCTCCCTCACTCGAAACACATCCATTACCCCGCTCGAGTCC  
GAAAATTTTGCAGTGCACCGTGATTTTTCTGGTGGGGTATCTTACCC  
GCCACTCGAGTCACGGATGCGCTTGCCCTGTTCCCAAAAACCTTACCAC  
CCTGTCGCGCACTACATGTCTTGACAGTCACTAACCCTGGACAATAGGAA  
GCCGCCGAGCTCGGAAAGGGTTCCTTCAAGTACGCCTGGGTTCCTTGACAA  
GCTCAAAGCCGAGCGTGAGCGTGGTATCACCATTGATATCGCTCTCTGGA  
AGTTCGAGACTCCTCGCTACTATGTCACCGTCATTGGTATGTTGTCAGT  
TCTCACACTATCATGTATTCATCATGCTAACATCTCTCTCAGATGCCCC  
GGTCATCGTGATTCATCAA

**> OKY1-2.1C1 (Fg034\_Ky2.1) (*F. tricinctum* NRRL 36147, MLST2-a)**

GTTCTTGATGAAATCACGATGACCGGGGGCATCTGAGAGAGATGTTAGCA  
TGATGAATACATGATAGTGTGAGACAGTGACAACATAACCAATGACGGTGA  
CATAGTAGCGAGGAGTCTCGAACTTCCAGAGAGCGATATCAATGGTGATA  
CCACGCTCACGCTCGGCTTTGAGCTTGTCAAGAACCCAGGCGTACTTGAA  
GGAACCCCTTTCCGAGCTCGGCGGCTTCCTATTGTCCAGTGGTTAGTGACT  
GCAAGACATGTAGTGCGCGACAGGGTGGTAAGGTTTTGTGGGAACAGGGC  
AAGCGCATCCGTGACTCGAGTGGCGGGGTAAAGATACCCACCCAGAAAAAA  
TCACGGTCGCACCGCAAAATTTTCGGACTCGAGCGGGGTAAATGGATGTGT  
TTCGAGTGAGGGAGCGAATCGTGGGAATCGATGGGAGCGCGCGTAATCGA  
AGGGATATTGACTAACCTTCTCGAACTTCTCGATGGTTCGCTTGTGATA  
CCACCGCACTGGTAGATCAAGTGACCGGTCTGTCAAAAGTTAGCAAGATG  
TTTTGGTGATACCCTGCCAGGTTTGGCGGATTCCGAGTGCAGATAAGCAA  
CCCGCTGTTGGTTGTACTTACAGTGGTCGACTTGCCAGAGTGCACGTGGC  
CGATGACGACGACGTTAAGG

**> OKY2-2.2A3 (Fg035\_Ky2.2) (*F. tricinctum* NRRL 36147, MLST2-a)**

ACTCACCTTAACGTCGTCGTCATCGGCCACGTCGACTCTGGCAAGTCGAC  
CACTGTAAGTACAACCAACAGCGGGTTGCTTATCTGCACTCGGAATCCGC  
CAAACCTGGCAGGGTATCACCAAAACATCTTGCTAACTTTTGACAGACCG  
GTCACCTTGATCTACCAGTGCGGTGGTATCGACAAGCGAACCATCGAGAAG  
TTCGAGAAGGTTAGTCAATATCCCTTCGATTACGCGCGCTCCCATCGATT  
CCCACGATTCGCTCCCTCACTCGAAACACATCCATTACCCCGCTCGAGTC  
CGAAAATTTTGCAGTGCACCGTGATTTTTCTGGTGGGGTATCTTACCC  
CGCCACTCGAGTCACGGATGCGCTTGCCCTGTTCCCAAAAACCTTACCA  
CCCTGTCGCGCACTACATGTCTTGACAGTCACTAACCCTGGACAATAGGA  
AGCCGCCGAGCTCGGAAAGGGTTCCTTCAAGTACGCCTGGGTTCCTTGACA  
AGCTCAAAGCCGAGCGTGAGCGTGGTATCACCATTGATATCGCTCTCTGG  
AAGTTCGAGACTCCTCGCTACTATGTCACCGTCATTGGTATGTTGTCAGT  
GTCTCACACTATCATGTATTCATCATGCTAACATCTCTCTCAGATGCCCC  
CGGTCATCGTGATTCATCAA

**> OKY2-2.2A1 (Fg036\_Ky2.2) (*F. graminearum*)**

CATGTTCTTGATGAAATCACGGTGACCGGGAGCGTCTGATAGCCATGTTA  
GTATGAGAATGTGATGACAGCAGTGGTGACAACATAACCAATGACGGTGAC  
ATAGTAGCGAGGAGTCTCGAACTTCCAGAGGGCGATATCAATGGTGATAC  
CACGCTCACGCTCGGCTTTGAGCTTGTCAAGAACCCAGGCGTACTTGAAG  
GAACCCTTACCGAGCTCGGCGGGCTTCCTATTGACAGGTGGTTAGTGACTG  
GTTGACACGTGATGATGAGCGCCCAGGGAATGGTTTGTGGGAAGAGGGCA  
GACGCCTGTCGCTCGAGTGGCGGGGTATGAGCCCCACCGGGGAAAAAATT  
ACGACAAAGCCGCAAAATTTTTGACCTCGAGCGGGGTAAACAGGCGCGTAT  
CGAGTCGTCGTGTGAGGGCGATTTCGAATGATATTTTCGAAAGGGAAAAGGG  
CGCGCGATCGAGGAAAATGAGACCAACCTTCTCGAACTTCTCGATGGTTC  
GCTTGTGCGATACCACCGCACTGGTAGATCAAGTGACCGGTCTATCAAAGT  
ATGTCAGCACATTGGAAATTTGAAACTACCCCGCCAAGTGTCGGCGGGGT  
TGGGATGCGGTGGTACTCACAGTGGTCGACTTGCCAGAGTCGACGTGGCC  
GATGACGACGACGTTAAGGT

**> OKY2-1.2B1 (Fg037\_Ky2.2) (*F. tricinctum* 97%)**

ACTCACCTTAACGTCGTCGTCATCGGCCACGTCGACTCTGGCAAGTCGAC  
CACTGTAAGTACAACCATGAGCGAGTCGCTTATCTGCACTCGGAACCCGC  
CAAACCTGGCGGGGTATCACCACAACATTTTGCTAACTTTTGACAGACCG  
GTCACCTTGATCTACCAGTGCGGTGGTATCGACAAGCGAACCATCGAGAAG  
TTCGAGAAGGTTAGTCAATATCCCTTCGATTACACGCGCGCTCCCATCGA  
TTCCCACGATTCGCTCCATCACTCGAAACGCATCCATTACCCCGCTCGAG  
CCCGAAAATTTTGCGGTGCGACCGTGATTTTTTCTGGTGGGGTATCTTA  
CCCCGCCACTCGAGTGACGGATGCGCTTGCCCTGTTCCCACAAAACCTTA  
CCGCCCTGTCGCGCACTACATGTCTTGCAAGTCACTAACCATTTCGACAATA  
GGAAGCCGCCGAGCTCGGAAAGGGTTCCTTCAAGTACGCCTGGGTCTTG  
ACAAGCTCAAAGCCGAGCGTGAGCGTGGTATCACCATTGATATCGCTCTC  
TGGAAGTTCGAGACTCCTCGCTACTATGTCACCGTCATTGGTATGTTGTC  
ACCGTCTCACACTATCATGCTTTCATCATGCTAACATCACTCTCAGATGC  
CCCCGGTCATCGTGATTTCA

**> OKY2-1.2B2 (Fg038\_Ky2.2) (*F. tricinctum* 97%)**

GTTCTTGATGAAATCACGATGACCGGGGGCATCTGAGAGTGATGTTAGCA  
TGATGAAAGCATGATAGTGTGAGACGGTGACAACATAACCAATGACGGTGA  
CATAGTAGCGAGGAGTCTCGAACTTCCAGAGAGCGATATCAATGGTGATA  
CCACGCTCACGCTCGGCTTTGAGCTTGTCAAGAACCCAGGCGTACTTGAA  
GGAACCCTTTCCGAGCTCGGCGGGCTTCCTATTGTCGAATGGTTAGTGACT  
GCAAGACATGTAGTGCGCGACAGGGCGGTAAGGTTTTGTGGGAACAGGGC  
AAGCGCATCCGTCCTCGAGTGGCGGGGTAAAGATACCCACAGAAAAAA  
ATCACGGTTCGACCGCAAAATTTTCGGGCTCGAGCGGGGTAAATGGATGCG  
TTTCGAGTGATGGAGCGAATCGTGGAATCGATGGGAGCGCGCGTGTAAT  
CGAAGGGATATTGACTAACCTTCTCGAACTTCTCGATGGTTCGCTTGTCG  
ATACCACCGCACTGGTAGATCAAGTGACCGGTCTGTCAAAAGTTAGCAAA  
ATGTTGTGGTGATACCCCGCCAGGTTTGGCGGGTTCGAGTGCAGATAAG

CGACTCGCTCATGGTTGTACTTACAGTGGTCGACTTGCCAGAGTCGACGTGGC  
CGATGACGACGACGTTA

b) Species specific and Chemotyping sequences for the Fusarium sp. strains isolated from FHB symptomatic wheat heads collected in Owensboro, KY and Hatfield, IN.

These sequences represent the sequences of Tri3 gene amplified from Owensboro, KY, and Hartfield, IN strains using chemotyping primers 3CON and 3D15A (Ward et al., 2002), and the fusarium graminearum sequences generated by Fg16f, and Fg16F primers (Demeke et al., 2005).

**> OKY2-2.1B1.1; Primer 3CON**

AAGACCTACGGACACACGTCCTCGCCTGTTGTAGTTCGCTTGATTTCGC  
GCGGAATGAAGCGTTCGTTGACACCGTCACTGATGAACAACTGTTGAA  
CGATTGTTAGCATTTTAGGGACGTAATTTACGAGATTTGGTAACCATA  
CCGGGTTTGCTTCTGTCCCTTCGAACGGATTTGGTTTGCTAGAGCGGTG  
TTAGCTGATGCGATGAGAGAGCAACTAGTTTCACTCACGCGTTCAAAT  
AACCGGCCTCGAAGTTATGGACTCTGAGGCCCAGAGCCTCCAGGAACG  
GATTTCCAAGCCATTGATCGTAACATTTCTTGATATCCCTGCAAGCCTT  
CTCTAGAGCTTTGACTTGAATCTCTTTTTCATCCTTGTGGCTCACTGCAA  
TAGACTTCAGATTCTCGACGCGAACAACAGCAGCAGTCTGACACATGG  
CGTAGTATTTGCTGGCTATATCCTCACGCAGCGGCTTGCGACCATCTAC  
TGATGTTGGTGATAAGAAGAATTCGTCGTCTGAAAGATCAGTAGGCTT  
GAGATGGTCCAGCAGAGCGAGTATGATGGCAGCTTGGGGGCAATACA  
ACGAGAT

*Gibberella zeae* genotype 15-ADON 15-O-acetyltransferase (Tri3) gene, partial cds

**>OKY2-2.1B2, Primer 3CON**

CTGGAAAGCGATCCCAGCAATGTTGAGAAAAGTTGATGTTCTCCGCCT  
CCACAGGAGTCAATTCAAAAAAATTCTTGGAAGAACAAGTTAGTGG  
TGGTAGAATTATCCAAAGAGCTGGAAGTCTGCTCCTTGAGTACCGCAG  
CATGCCTCTTCTTCTGCTCCTCCATATCGGCTAGAGAGAAAGCGATGGC  
AGCTTGGAATAAAAAA

No significant similarity found.

**>OKY2-2.2A3, Primer 3CON**

GGGGAAAAACGGCTCTAGAACAATGGGTTCTAATAGAGCGTTCTTGTC  
AATGAATGGAGGATGATCATATCTACCTAACATGTCTTTCACAGACCT  
GAGTAGATGCGGGAAGGATACAAAACAAAAGAAAACAAAAAAACAT  
GTCCAAACGCAGGGGTGCTTTCTACTGACAGCGGATGCATGGCTGTCA  
GTTAATGTGGATAGATCCTTGTCACACTACTTTGTCTGTATCGTGTTGC  
TCCAAACTGTGAACACCTGCATATCATGTCCTTGACGGCTTCACCGAAA  
TGTGGTCAATTCTTTCTCTGTATTTATTTGATGGGGCTGTTTGAGAAGA  
TCCAGGGTCTTCCGTCACCTTGTGTCTAGCGATGCTGTGGGGGATTAT

ACGATCTAAACAGCGTGATGGCAGCTTGGGGTCAGTATAACGCTACTTT  
ATTGATGGCAGCTTGGGGTCAGTAAAACGAGAGGGGGGGGGGGGAAAA  
TAAACATAGTTATTTCTTTTCCGATT

**No significant similarity found.**

**>PH-1, Primer 3CON**

AAGCCCTACGGACACACGTCCTCGCCTGTTGTAGTTCGCTTGATTTTCGC  
GCGGAATGAAGCGTTCGTTGACACCGTCACTGATGAACAACCTGTTGAA  
CGATTGTTAGCATTTTAGGGACGTAATTTACGAGATTTGGTAACCATTA  
CCGGGTTTGCTTCTGTCCCTTCGAACGGATTTGGTTTGCTAGAGCGGTG  
TTAGCTGATGCGATGAGAGAGCAACTAGTTTCACTCACGCGTTCAAAT  
AACCGGCCTCGAAGTTATGGACTCTGAGGCCCAGAGCCTCCAGGAACG  
GATTTCCAAGCCATTGATCGTAACATTTCTTGATATCCCTGCAAGCCTT  
CTCTAGAGCTTTGACTTGAATCTCTTTTTTCATCCTTGTGGCTCACTGCAA  
TAGACTTCAGATTCTCGACGCGAACAAACAGCAGCAGTCTGACACATGG  
CGTAGTATTTGCTGGCTATATCCTCACGCAGCCACTTGCGACCATCTAC  
TGATGTTGGTGATAAGAAGAATTCGTCTGAAAGATCAGTAGGCTT  
GAGATGGTCCAGCAGAGCGAGTATGATGGCAGCTTGGGGTCAGTAA

**No significant similarity found.**

**>3-ADON CONTROL (3-ADON28-102), Primer 3CON**

AACATTACGGAAAAACGTCCTCGCCTGTTGCAGTTCGCTTGATTTTCGCG  
AGGAATGAAGCGTTCGTTGACACCGTCACTGATGAACAACCTGTTGCAT  
GATTGTTAGTATTTACAGGGACGTAATTTACGAGATTCAGTGGCCATAC  
CGGGTTCGCTTCTGTTCCCTTCGAACGGATTCGGTTTCCTAAAGTGGTCA  
TGTGTTAGCCAATGCGA

*Gibberella zeae* genotype 3-ADON 15-O-acetyltransferase (Tri3) gene, partial cds

**>HIN1-1.2B4, Primer 3CON**

AGGGCTTACGGGACACACGTCCTCGCCTGTGTGTAGTTCGCTTGATTTTC  
GCGCGGAATGAAGCGTTCGTTGACACCGTCACTGATGAACAACCTGTTG  
AACGATTGTTAGCATTTTAGGGACGTAATTTACGAGATTTGGTAACCA  
TACCGGGTTTGCTTCTGTCCCTTCGAACGGATTTGGTTTGCTAGAGCGG  
TGTTAGCTGATGCGATGAGAGAGCAACTAGTTTCACTCACGCGTTCAA  
ATAACCGGCCTCGAAGTTATGGACTCTGAGGCCCAGAGCCTCCAGGAA  
CGGATTTCCAAGCCATTGATCGTAACATTTCTTGATATCCCTGCAAGCC  
TTCTCTAGAGCTTTGACTTGAATCTCTTTTTTCATCCTTGTGGCTCACTGC  
AATAGACTTCAGATTCTCGACGCGAACAAACAGCAGCAGTCTGACACAT  
GGCGTAGTATTTGCTGGCTATATCCTCACGCAGCCACTTGCGACCATCT  
ACTGATGTTGGTGATAAGAAGAATTCATCGTCTGAAAGATCAGTAGGC  
TTGAGATGGTCCAGCAGAGCGTTGTGATGGCCCTGCTGGCCTCGTGAT  
CACCGTCTCGCCTGTTGTGTTCTGTTGTTTCGCGTGGAATGAATGTTCTG  
GAACCGTCCTGATGAAAACCTGTTGACGATTGTTATATTTTAGGACTATT

TCCGAGATTGGTAACCTACTGGTTGCTTCGTCTTCGAAGGATTGGTTTG  
CAAGGGTTTACTGATGGTGAGAGACATTTTTCTCCCGTCAAAACGCCTC  
ATTTTGGATCGAAGCA

*Gibberella zeae* genotype 15-ADON 15-O-acetyltransferase (Tri3) gene, partial cds

>OKY2-2.2A3, Primer 3D15A

GCATCCGTTATCCCCACAGCATCGCTAGACAACAAAGTGACGGAAGAC  
CCTGGATCTTCTCAAACAGCCCCATCAAATAAATACAGAGAAAGAATT  
GACCACATTTTCGGTGAAGCCGTCAAGGACATGATATGCAGGTGTTTAC  
AGTTTGGAGCAACACGATACAGACAAAGTAGTGTGACAAGGATCTATC  
CACATTAAGTACAGCCATGCATCCGCTGTCAGTAGAAAGCACCCCTG  
CGTTTCGACATGTTTTTTTTGTTTTCTTTTGTGTTTGTATCCTTCCCGCATC  
TACTCAGGTCTGTGAAAGACATGTTAGGTAGATATGATCATCCTCCATT  
CATTGACAAGAACGCTCTATTAGAACCCATTTGTTTCTAGAGCCACTGC  
GTCACCTCCACTTCGTGAACCAGTCTTTGCCAAAAAAA

No significant similarity found.

>PH-1, Primer 3D15A

CGCCGGGACTCTCAGCCTACTGATCTTTCAGACGACGAATTCTTCTTAT  
CACCAACATCAGTAGATGGTCGCAAGTGGCTGCGTGAGGATATAGCCA  
GCAAATACTACGCCATGTGTCAGACTGCTGCTGTTGTTTCGCGTCGAGA  
ATCTGAAGTCTATTGCAGTGAGCCACAAGGATGAAAAAGAGATTCAAG  
TCAAAGCTCTAGAGAAGGCTTGCAGGGATATCAAGAAATGTTACGATC  
AATGGCTTGGAAATCCGTTCTGAGGCTCTGGGCCTCAGAGTCCATA  
ACTTCGAGGGCCGGTTATTTGAACGCGTGAGTGAACTAGTTGCTCTCTC  
ATCGCATCATCTAACACCGCTCTAGCAAACCAAATCCGTTTCGAAGGGA  
CAGAAGCAAACCCGGTATGGTTACCAAATCTCGTGAAATTACCTTTTTT  
ATATGCTAACAAATCGTTCAAAAGTTGTTCAAAAAGGAGAGGGTGTC

*Gibberella zeae* genotype 15-ADON 15-O-acetyltransferase (Tri3) gene, partial cds

>3-ADON CONTROL (3-ADON28-102), Primer 3D15A

ATTTTTTCAATACCGAAGCCAGTATTACAGAACAGGAATAGAGTAAAT  
ACCTTAAAAAGGTGATCACCATATTACTAAGCTATCGAAGTTTTTCTCCT  
AAAAATGAAGATATTGTATATAGAAAGGTAAGACCTTTTATACTCATC  
TAT

No significant similarity found.

>HIN1-1.2B4, Primer 3D15A

CCGGTCCTGACTCTCAGCCTACTGATCTTTCAGACGACGAATTCTTCTT  
ATCACCAACATCAGTAGATGGTCGCAAGTGGCTGCGTGAGGATATAGC  
CAGCAAATACTACGCCATGTGTCAGACTGCTGCTGTTGTTTCGCGTCGAG  
AATCTGAAGTCTATTGCAGTGAGCCACAAGGATGAAAAAGAGATTCAA  
GTCAAAGCTCTAGAGAAGGCTTGCAGGGATATCAAGAAATGTTACGAT

CAATGGCTTGGAAATCCGTTCTGAGGCTCTGGGCCTCAGAGTCCAT  
AACTTCGAGGCCGGTTATTTGAACGCGTGAGTGAACTAGTTGCTCTCT  
CATCGCATCAGCTAACACCGCTCTAGCAAACCAAATCCGTTCTGAAGGG  
ACAGAAGCAAACCCGGTATGGTTACCAAATCTCGTGAAATTACGTCCC  
TAAAATGCTAACAATCGTTCAACAGTTGTTTCATCAGTGACGGTGTCAA  
CGAACGCTTCATTCCGCGCGAAATCAAGCGAACTACAACACGCGAGGA  
CGTGTGTCCGTTGAGAGCATAGACTAAGTAAAAAAAAAAGTCTTTTT  
TGCTAC

*Gibberella zeae* genotype 15-ADON 15-O-acetyltransferase (Tri3) gene, partial cds

>OKY2-2.1B1.1, Primer 3D15A

CGTCCGGGGCCATCTCAAGCCCTACTGATCTTTCAGACGACGAATTCTT  
CTTATACCAACATCAGTAGATGGTCGCAAGTGGCTGCGTGAGGATAT  
AGCCAGCAAATACTACGCCATGTGTTCAGACTGCTGCTGTTGTTTCGCGTC  
GAGAATCTGAAGTCTATTGCAGTGAGCCACAAGGATGAAAAAGAGATT  
CAAGTCAAAGCTCTAGAGAAGGCTTGCAGGGATATCAAGAAATGTTAC  
GATCAATGGCTTGGAAATCCGTTCTGAGGCTCTGGGCCTCAGAGTC  
CATAACTTCGAGGCCGGTTATTTGAACGCGTGAGTGAACTAGTTGCT  
CTCTCATCGCATCACCTAACACCGCTCTAGCGAACC AAATCCGTTTTAA  
GGGACACAGGCAAACCC

*Gibberella zeae* genotype 15-ADON 15-O-acetyltransferase (Tri3) gene, partial cds

>OKY2-2.1B2, Primer 3D15A

GGGGACCCCCCTCCCTCGTCTCTTGCTTATCCCCCGCCATCGACTAA  
AACGCATCACAGGCCTGATCGCCCCCTCGAGGCATCCCCTCATGGGAT  
GCGCACCAGCTGGATACTCTTGTTGTGTTTCGTGTTCTTCTCCATCTGTGC  
GTAAAAGTTGACTGTACTACCAAGGCGGGGGGACGCCCCCTCATGATG  
GCAGCTTGGGTCAGTAAA

No significant similarity found.

>PH-1, Primer Fg16F

CTTTCTGAATGTCTTGAGGCATGCGAACTTTTGAGGGCTTGCGTTGCTC  
ATGGATGATCTGGATATGCTTCGCCATATTGCCCTTTGTCCCGAATGAC  
TTGGGGCAATGGGGACATTTGAAGGGCTGGCGCTTCTCGTGAACACAC  
GAGATATGTCTCTTCAAGTCTTTCTTTAATTGGAATGCCTTGGGGCAAG  
TCGGGCATTTCGTGCTGTTTAGCCATCTTGCGATCGGTGCCTGCCAAACA  
ATAGCAGTGTAGAACAGTCGGTTAAGGATAATAGCAGTGTAGAATAGT  
CGGTTAAGGTTGCTGCAGGTGGAAACAGATGACAAGATTCAGGCACAT  
ATAACTGCACTTATAAGTTTGCCATGTTCGGATACCTACCC

*Gibberella zeae* strain 02-11 strain specific genomic sequence



**>3-ADON CONTROL (3-ADON28-102), Primer Fg16F**

CTTGTCTTGAAAGTCTTTGAAGCATGCGAACTTTTGAAGGGCTTGCGT  
TGCTCATGGATGATCTGGATATGCTTCGCCATATTGCCCTTTGTCCCGA  
ATGACTTGGGGCAATGGGGACATTTGAAGGGCTGGCGCTTCTCGTGAA  
CACACGAGATATGTCTCTTCAAGTCTTTCTTTAATTGGAATGCCTTGGG  
GCAAGTCGGGCATTTCGTGCTGTTTAGCCATCTTGCGATCGGTGCCTGCC  
AAACAATAGCAGTGTAGAACAGTCGGTTAAGGATAATAGCAGTGTAG  
AATAGTCGGTTAAGGTTGCTGCAGGTGGAAACAGATGACAAGATTTCAG  
GCACATATAACTGCACTTATAAGTTTGCCATGTCGGATACCTACCCAGA  
*Gibberella zeae* strain 02-11 strain specific genomic sequence

**>HIN1-1.2B4, Primer Fg16F**

CTGTCTGAAAGTCTTGAGGCATGCGAACTTTTGAAGGGCTTGCGTTGCT  
CATGGATGATCTGGATATGCTTCGCCATATTGCCCTTTGTCCCGAATGA  
CTTGGGGCAATGGGGACATTTGAAGGGCTGGCGCTTCTCGTGAACACA  
CGAGATATGTCTCTTCAAGTCTTTCTTTAATTGGAATGCCTTGGGGCAA  
GTCGGGCATTTCGTGCTGTTTAGCCATCTTGCGATCGGTGCCTGCCAAAC  
AATAGCAGTGTAGAACAGTCGGTTAAGGATAATAGCAGTGTAGAATAG  
TCGGTTAAGGATAATAGCAGTGTAGAATAGTCGGTTAAGGTTGCTGCA  
GGTGGAAACAGATGACAAGATTTCAGGCACATATAACTGCACTTATAAG  
TTTGCCATGTCGGATACCTACCC  
*Gibberella zeae* strain 02-11 strain specific genomic sequence

**>OKY2-2.1B1.1, Primer Fg16F**

CCTTGGCCGAAAAGTCTTGAGGCATGCGAACTTTTGAAGGGCTTGCGTT  
GCTCATGGATGATCTGGATATGCTTCGCCATATTGCCCTTTGTCCCGAA  
TGACTTGGGGCAATGGGGACATTTGAAGGGCTGGCGCTTCTCGTGAAC  
ACACGAGATATGTCTCTTCAAGTCTTTCTTTAATTGGAATGCCTTGGGG  
CAAGTCGGGCATTTCGTGCTGTTTAGCCATCTTGCGATCGGTGCCTGCCA  
ACAATAGCAGTGTAGAACAGTCGGTTAAGGATAATAGCAGTGTAGA  
ATAGTCGGTTAAGGTTGCTGCAGGTGGAAACAGATGACAAGATTTCAGG  
CACATATAACTGCACTTATAAGTTTGCCATGTCGGATACCTACCC  
*Gibberella zeae* strain 02-11 strain specific genomic sequence

**>OKY2-2.1B2, Primer Fg16F**

GTAGGGTCCGAATTTGAAAGAAAACACGGGACTTCTCCATACACGGCT  
ACAGGGTAGCTCTTGTTCAAAATAGTGTACTTCTATAAATTTGTTGCCA  
TCAAACCCATAAATTATATGAGATAGTAAACGAGATATATAGATACTG  
TTTATATGTTCCCTAATCGCCGACTGGATGGTTAATCATTCGGCGTTTGG  
GGTCGTTCTGGCGGTTGACTGTCTATACCTACACGCGGACGACTCCGAC  
CCCTCCGGCGATACTGGCAAGTCTGTTTTCTACTTCTCCGTTCAAAAC  
TTTATCAAGTGTTTCATCCTTGCGGTTTTTCGCCGTCAAGCATAGCCGTTG  
GTCAGTAAGAACAAGGCAACCGGACCGCGACTCAGCTCCCCTGACTTG

TCTTGCATTTACGATAGACCCACCATAGTCGAGGTTCTCGGTCAATTC  
TTTGAGCTTCCATACCCGAAACCACTTACACGGCGCCCCTCTTCGGTTT  
GATCTGCCATGTCGGATACCTACCCAAAA

**No significant similarity found.**

**>OKY2-2.2A3, Primer Fg16F**

ACAGGGTTCGTTTCGCCTGTATGTAGTTCGCTTGATCCTAGCGGAAAGA  
AGCGTCGTTCAAGCGAAAAAGAGGCCAGGGTTGACGTTGCAATTTTTG  
CTGAAATAAAGACGACCATTTGGTACCAAGGGGCAGTCGCCTTAGTCG  
TTTGTGTCCATGCCCCCATTATT

**No significant similarity found.**

**>3-ADON CONTROL (3-ADON28-102), Primer Fg16R**

AAGGGGCTTTAATGGTGCCTGAATCTTGTTCATCTGTTTCACCTGCAGCA  
ACCTTAACCGACTATTCTACACTGCTATTATCCTTAACCGACTGTTCTA  
CACTGCTATTGTTTGGCAGGCACCGATCGCAAGATGGCTAAACAGCAC  
GAATGCCCCGACTTGCCCCAAGGCATTCCAATTAAAGAAAGACTTGAAG  
AGACATATCTCGTGTGTTTCACGAGAAGCGCCAGCCCTTCAAATGTCCC  
CATTGCCCCAAGTCATTTCGGGACAAAGGGCAATATGGCGAAGCATATC  
CAGATCATCCATGAGCAACGCAAGCCCTTCAAAGTTTCGCATTGCCTC  
AAGACATTCTCAGACCAGAGCAATTTGACGCAACATATCCGGAG

***Gibberella zeae* strain 02-11 strain specific genomic sequence**

**>HIN1-1.1.2B4, Primer Fg16R**

AAGGCGTTAATGTGCCTGATCTTGTTCATCTGTTTCCACCTGCAGCAACC  
TTAACCGACTATTCTACACTGCTATTATCCTTAACCGACTATTCTACAC  
TGCTATTATCCTTAACCGACTGTTCTACACTGCTATTGTTTGGCAGGCA  
CCGATCGCAAGATGGCTAAACAGCACGAATGCCCCGACTTGCCCCAAGG  
CATTCCAATTAAAGAAAGACTTGAAGAGACATATCTCGTGTGTTTCACG  
AGAAGCGCCAGCCCTTCAAATGTCCCCATTGCCCCAAGTCATTTCGGGA  
CAAAGGGCAATATGGCGAAGCATATCCAGATCATCCATGAGCAACGCA  
AGCCCTTCAAAGTTTCGCATTGCCTCAAGACATTCTCAGACCAGAGCA  
ATTTGACGCAACATATCCGGAGAACCGCTCGTTGGCTGCGCTTTTGT  
TAGAAGAGTTCCTAATACTATCCCCTAAAAGAGGGGGGGGGGGGTAAAC  
AGTA

***Gibberella zeae* strain 02-11 strain specific genomic sequence**

**>OKY2-2.1B1.1, Fg16R**

TAATGTGGCTAATGTGCCTGATCTTGTTCATCTGTTTCCACCTGCAGCAA  
CCTTAACCGACTATTCTACACTGCTATTATCCTTAACCGACTGTTCTAC  
ACTGCTATTGTTTGGCAGGCACCGATCGCAAGATGGCTAAACAGCACG  
AATGCCCCGACTTGCCCCAAGGCATTCCAATTAAAGAAAGACTTGAAGA  
GACATATCTCGTGTGTTTCACGAGAAGCGCCAGCCCTTCAAATGTCCCC

ATTGCCCCAAGTCATTTCGGGACAAAGGGCAATATGGCGAAGCATATCC  
AGATCATCCATGAGCAACGCAAGCCCTTCAAAAGTTCGCATTGCCTCA  
AGACATTCTCAGACCAGAGCAATTTGACGCAACATATCCGGAGGAAAA  
*Gibberella zeae* strain 02-11 strain specific genomic sequence

**>OKY2-2.2B2, Primer Fg16R**

AAAACGGACGAGGAGCGTCGTCTTCAAGCGACAACGAGATGTTGTCTG  
CGCATTTACTCGAGAAAGGTCTAGGTCTGGATCTGGATGCGCAACCAG  
CTGCGCGCGACGGGATGCATCTACGACAAGTCTTCGCCAATAGGGTCA  
TGCCAGTGGCATTCGCCCTGCCGTGGATCGCCGGCATGTTTCATATCCTG  
GATCCCTTTACAAACATCAGCGTATCCAACGCCCCAGATTTCTGTAA  
GTCTCGACACCATTCAATTGCGCCGCTGTCGCATAGAGACATATCTAAC  
CCGCGGTAGTATTCTGGCTGCAATCATCAGCACCTAGAAAGTCCTCAG  
CATCATCGCCGTATTCATCTACACGTCCCTCCGCCCCGATGTCCTCGAC  
AACGAATCCTTCCGCGGCGTCACACTAAAAGAAAAGGCCCTTCGCGCC  
TGGTTCTGGTTCCTCTTCATCAGCCCGTTCGTTACAGGCACCATGGTCG  
TCCTCATGGCCAGCGACATTTCTGCGACCCGGAGGACGACCTGGACA  
TGCACCAGCCCATCTGCCATGTCGGATACCTACCCCAAATA

No significant similarity found.

**>OKY2-2.2A3, Primer Fg16R (88% identity)**

AAAGCCCGATGGAAAAAGCGTCGTTCTTCAAAGCGACAACGAAATGTC  
GTCTGCGCATTTACTCGAGAAAGGTCTAGGTAGGATCTGGATGCGCAA  
CCAGCTGCGCGCGACGGGATGCATCTACAAAAAGTCTTCGCCAATAGG  
GTCATGCCAGTGGCATTCGCCCTACCGTGGATCGCCGGCATGTTAATAT  
CCTGGATCCCTTTTCGCAAATATCAGCGTCTCCAACGCCCCAGATTTCT  
GTAAGTCTAGACGCCTTTCAATTGCGCCGCGTTCGCATAGACATATCTA  
ACCCGCGGTAGTATTCTGGCTGCAGCCATCAGCACTCTAAAAGTCCTC  
AGCATCATCGCCGTCTTCATCTACACGTCCCTCCGCCCCGAGGCCCTCG  
ACAACGAATCTTTCCGCGGCGTCACACTAAAAGAAAAGGCCCTGCGTT  
TTTGGTTCTGGTTCCTCTTCATCAGCCCTTTCCACACGGGCACCATGGT  
CGA

*Nectria haematococca* mpVI 77-13-4 hypothetical protein, mRNA

**>PH-1, Primer Fg16R**

AATGGCGTTAATGTGCCTGATCTTGTCATCTGTTTCCACCTGCAGCAAC  
CTTAACCGACTATTCTACACTGCTATTATCCTTAACCGACTGTTCTACA  
CTGCTATTGTTTGGCAGGCACCGATCGCAAGATGGCTAAACAGCACGA  
ATGCCCCGACTTGCCCCAAGGCATTCCAATTAAAGAAAGACTTGAAGAG  
ACATATCTCGTGTGTTACAGAGAAGCGCCAGCCCTTCAAATGTCCCCAT  
TGCCCCAAGTCATTCGGGACAAAGGGCAATATGGCGAAGCATATCCAG  
ATCATCCATGAGCAACGCAAGCCCTTCAAAAGTTCGCATTGCCTCAAG  
ACATTCTCAGACCAGAGCAATTTGACGCAACATATCCGGAG

*Gibberella zeae* strain 02-11 strain specific genomic sequence

## APPENDIX III

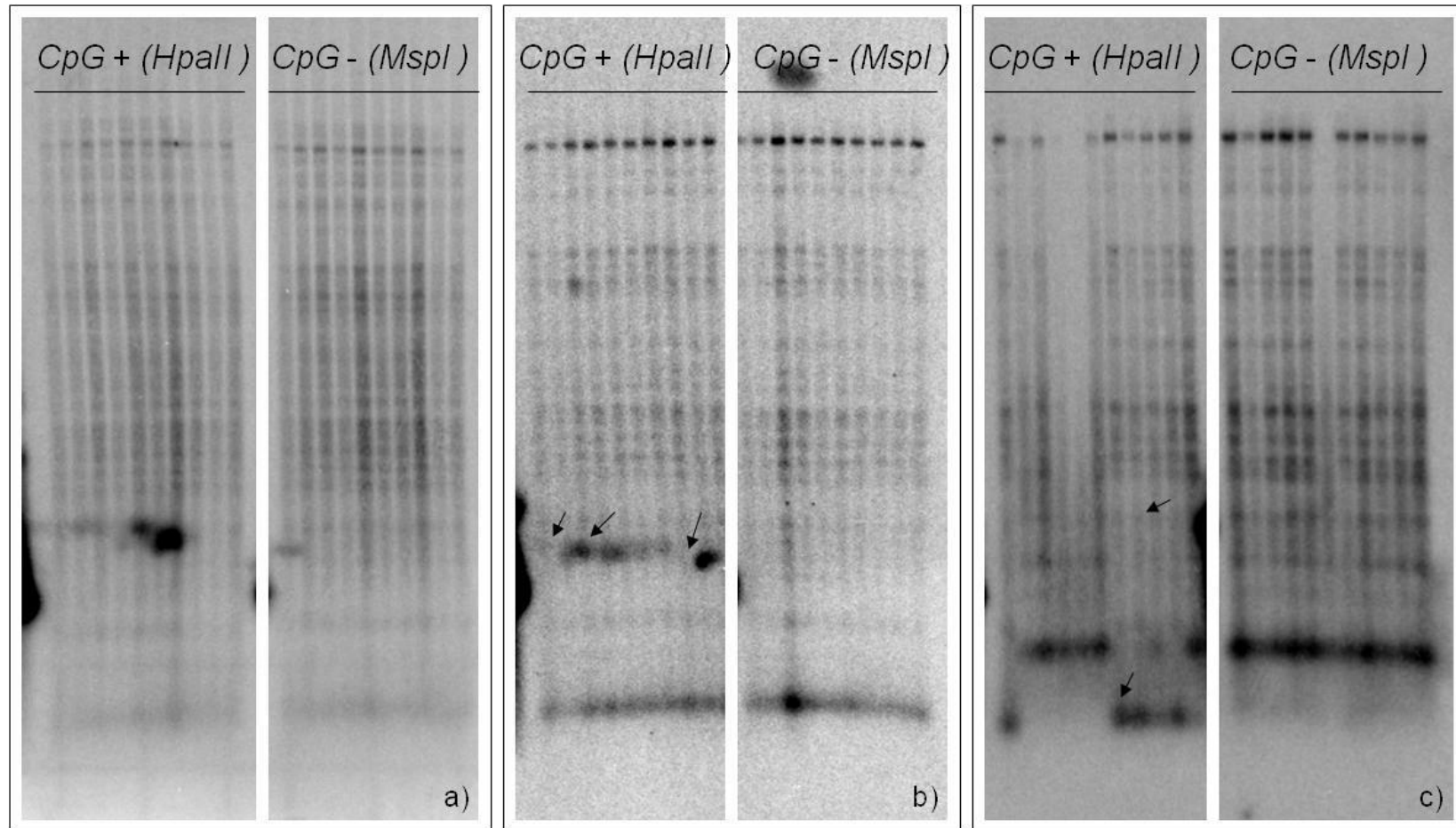
### Detection of methylation in strains derived from from crossing.

I was surprised to discover that strains derived from ascospore progeny of selfing in *G. zae* displayed a wide range of variation in aggressiveness and other traits, when compared with strains derived from single conidia. One explanation for this could be the presence of epigenetic factors that affect gene expression in the meiotic progeny. One possibility for an epigenetic factor could be DNA methylation. There are three major types of DNA methylation: CpG<sup>m</sup> CG, *dam* GmATC, and *dcm* C<sup>m</sup>CWGG. One of the ways to test for the presence of DNA methylation is to restrict the DNA with an isoschizomer pair of REs, one of which is sensitive, and the other insensitive to a specific methylation status. The *BsoBI/AvaI* isoschizomer pair is used to detect CpG methylation with *BsoBI* being the insensitive, and *AvaI* the sensitive isoschizomer. The presence of *dam* methylation is tested by the use of *Sau3AI/MboI* isoschizomer pair. In this pair *Sau3AI* is the insensitive, and *MboI* is the sensitive enzyme. I decided to use this method to test a group of progeny of a cross between PH-1 and Gz3639, for the presence of methylation, and for evidence that methylation patterns were altered during meiosis.

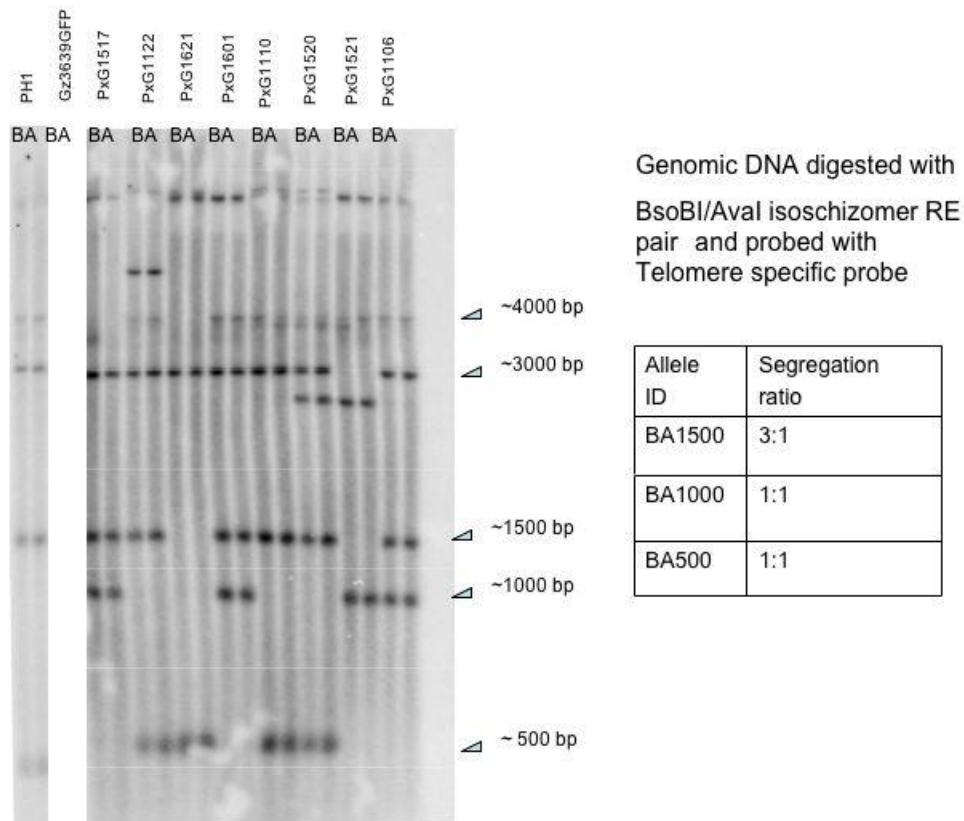
#### Results:

I tested the strains for the presence of CpG (Figure 1 and Figure 2) and some also for *dam* (Figure 3) methylation. I found no evidence for CpG methylation in any strain, In the hetherothallic outcrossed progeny I found evidence only *dam* methylation. I found that patterns of methylation appeared to vary in the progeny (Figure 1). These are only preliminary data and until more work is done, it would not be possible to say that the variability I observed was related to epigenetic factors vs recombination.

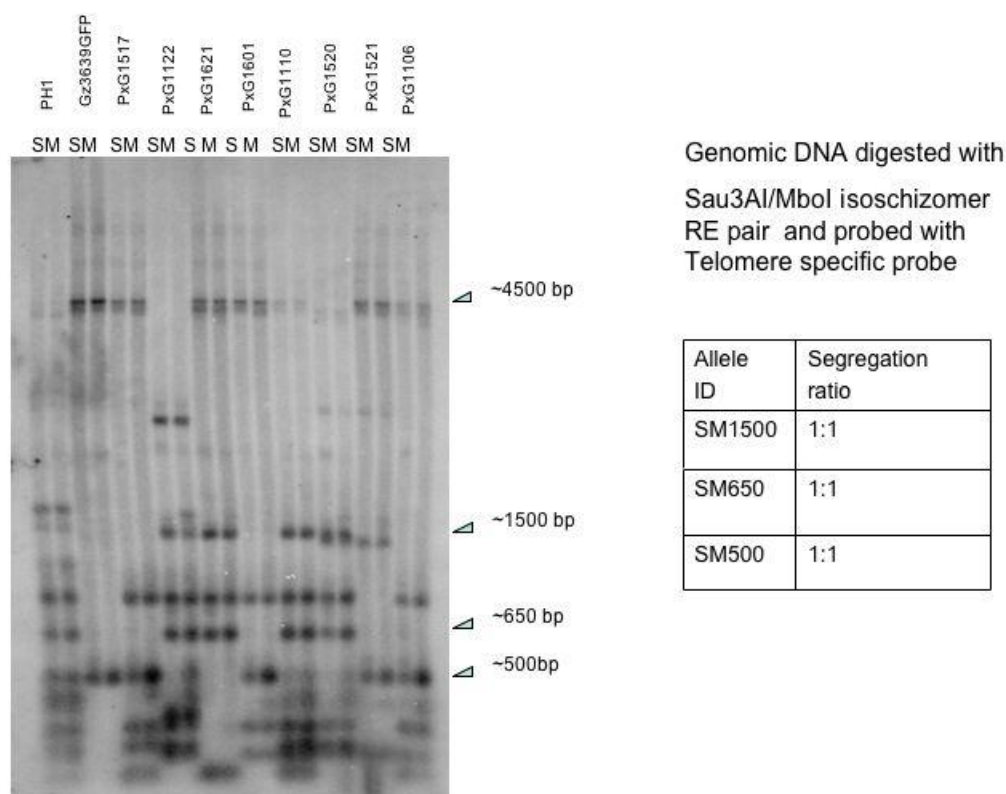
These experiments should be repeated with mitotic and meiotic selfed progeny.



**Figure 1.** Detection of CpG DNA methylation patterns in mitotic (a), meiotic homothallic (b), and meiotic heterothallic (c) *F. graminearum* strains. The genomic DNA was digested with CpG sensitive (*HpaII*) and CpG insensitive (*MspI*) RE and probed with telomere specific (TTAGGG)<sub>n</sub> probe.



**Figure 2:** Analysis of DNA CpG methylation status of the recombinant PxG progeny sets. The genomic DNA was digested with *BsoBI* and *AvaI* isoschizomer pair and probed with telomere probe. There is no difference in the digestion pattern detected by comparison of *BsoBI* and *AvaI* restriction patterns.



**Figure 3 :** Analysis of DNA *dam* methylation status of the recombinant PxG progeny sets. The genomic DNA was digested with *Sau3AI* and *MboI* isoschizomer pair and probed with telomere probe. The differences in restriction pattern between the *dam* methylation insensitive (S = *Sau3AI*) and *dam* methylation sensitive (M = *MboI*) are indicated with black arrows.

## VITA

Sladana Bec was born August 8, 1977 in Kutina, Croatia. She grew up on the family farm in Trnoviticki Popovac, Croatia. In 2001 she was awarded B.S. in Horticulture and Landscape Design from the University of Zagreb, Croatia. Between 1998 and 2001 Bec held the position of research assistant in mycology under guidance of Prof. Dr. Romano Bozac. In 2005 she was awarded MS degree in Plant and Soil Sciences majoring in plant breeding mentored by Dr. Todd W. Pfeiffer. In the fall of 2005 she joined the Department of Plant Pathology to pursue PhD degree working in the lab of Dr. Lisa J. Vaillancourt.

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Sladana Bec